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B_{12}-*retro*-Riboswitches: Guanosyl-Induced Constitutional Switching of B_{12} Coenzymes

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Dedicated to Professor Dieter Seebach on the occasion of his 70th birthday

Abstract: Complete B_{12} derivatives are natural "molecular switches" as a result of the coordinative switch ("base on" or "base off") of the natural nucleotide base. Certain predesigned B_{12}–nucleotide conjugates were shown recently to behave as "*retro* riboswitches", in which the nucleotide environment modified the equilibrium between these two isomeric B_{12} structures. In contrast, the "reverse" situation has been discovered in natural B_{12} riboswitches, in which the binding of coenzyme B_{12} induces a conformational switch in the RNA species. The first (predesigned) B_{12}-*retro*-riboswitches were DNA conjugates of methylcobalamin. We describe herein two representative B_{12}-*retro*-riboswitches, in which an appended (RNA) nucleotide is used to destabilize the base-on form and induce the base-on to base-off switch. Through use of heterogeneous solid-phase synthetic methods, Co_β–cyanocobalamin-(3′→2′)-2′-methoxyguaninyl-3′-ate was prepared first as the crucial covalent RNA conjugate of vitamin B_{12}. This cyanocorrinoid opened the door to two organometallic B_{12}–nucleotide conjugates, which were made by electrosynthetic means: the cyanocorrinoid was cleanly methylated or adenosylated at the cobalt center to furnish covalent RNA conjugates of the organometallic B_{12} cofactors methylcobalamin and coenzyme B_{12}, respectively. At room temperature, aqueous solutions of both of these organometallic RNA–B_{12} conjugates exhibited properties indicative of significant weakening of the axial (Co–N) bond (of their base-on forms) and of an enhanced formation of the base-off species. The base-on to base-off switch was studied by UV/Vis and NMR spectroscopic studies, which showed that the switch was very temperature-dependent and was accentuated with increasing temperatures. Thermodynamic data of the two organometallic RNA–B_{12} conjugates revealed an important contribution of entropic effects to the observed base-on to base-off switch. The two organometallic RNA–B_{12} conjugates thus acted as B_{12}-*retro*-riboswitches and allowed the observation of a temperature-dependent reverse switch in the B_{12} cofactor moiety, induced by the appended nucleotide moiety. This behavior may be of interest in the "RNA-world" hypothesis, in which (simple) B_{12} derivatives are thought to act as possible catalytic enhancers ("cofactors") in RNA-based "B_{12} ribozymes".

Keywords: cobalamins · molecular switches · organometallic chemistry · RNA · vitamins

Introduction

B_{12} derivatives are vitamins in humans and many other organisms, and they are available from microorganisms, as only these have mastered the required biosynthesis.[1–3] Vitamin B_{12} (cyanocobalamin; **1**) is one of the most complex and fascinating natural products.[4,5] In **1**, an exceptional 5,6-dimethylbenzimidazolyl (DMB) α-pseudonucleotide is appended to a no less unique cobalt–corrin unit.[5,6] The DMB base features an intramolecular cobalt coordination to the lower (α) axial position of the cobalt center in **1**, as in the "base-on" form of the two mammalian B_{12} cofactors methylcobalamin (**2**)[6,7] and adenosylcobalamin (coenzyme B_{12}; **3**).[6,8]

Binding of B_{12} cofactors to their cognate apo-enzymes is accompanied by a remarkable mutual structural adaptation of the corrinoid and protein parts.[9,10] Methyltransferases, which depend upon **2** and other methylcorrinoids, have been shown to bind their methylcorrinoid cofactor in their "base-off" form, which is not directly observable.[10–12] This (pro-

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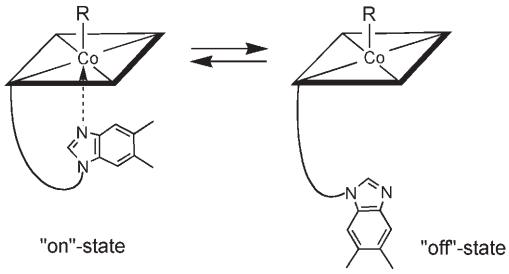
tein-induced) constitutional restructuring of bound B_{12} has been suggested to be a way that the (frequently) multimodular enzymes tune the reactivity of the corrinoid[13,14] and control its orientation according to its task(s) as a cofactor in methyltransferases.[10] In enzymes dependent upon coenzyme B_{12} (**3**), the situation is even more puzzling and the bound cofactor **3** has sometimes been observed to be base on,[15–17] but (so far) more frequently to be base off.[18,19] In this latter group of enzymes, a histidine substituted for the DMB base at the corrin-bound cobalt center and the B_{12} cofactor **3** was thus observed in its "base-off/his-on" form.[18,19]

B_{12}-dependent organisms have developed a sophisticated repertoire for the effective uptake, controlled transport,[20] metabolic transformation,[3] and use as cofactors of the scarcely available corrinoids (e.g., see refs [21–23]) as well as for the genetic regulation of the proteins involved in B_{12}-dependent processes and B_{12} biosynthesis.[24] Recently, "B_{12} riboswitches" were discovered to be another element in the complex regulation of proteins relevant in B_{12} metabolism.[25–27] B_{12} riboswitches are now known to be widely occurring control elements in the 5′-untranslated region of natural messenger RNA species, which bind coenzyme B_{12} (**3**) and then undergo a conformational switch. This structural change of the 5′-untranslated region of the mRNA leads to inhibition of the expression of proteins relevant in B_{12} biosynthesis or B_{12} transport.[25–27] These findings point to a new mechanism of genetic control[28] and establish the biological relevance of the direct interaction between RNA and B_{12} coenzymes. Artificial RNA sequences were prepared in chemical evolution experiments in vitro and can bind small molecules tightly, including vitamin B_{12}, (i.e., acting as aptamers).[29–31] Crystallographic analysis revealed a detailed structure of the B_{12} aptamer[32] and helped to rationalize its high affinity for vitamin B_{12}.[29]

Binding coenzyme B_{12} (**3**) to B_{12}-binding riboswitches is thus accompanied by a critical conformational adaptation of the RNA portion. The question of how corrinoid **3** is bound by this mRNA is unanswered. We became interested in the structural aspect of how and whether a functional RNA environment (as encountered in the B_{12} riboswitches) produces structural changes in a bound B_{12} cofactor.[33,34] Indeed, natural "complete" cobamides contain a variety of cobalt-coordinating nucleotide moieties,[35] which may be seen as possible remnants of the interaction of corrinoids with a nucleotide environment.[34] The natural B_{12}–nucleotide moiety provides the B_{12} cofactors with the remarkable ability to be a "molecular switch" that may shuttle between the two unique constitutional isomers,[36] frequently described as base-on and base-off forms of B_{12} (Scheme 1).[34] At the same time, through its ability to coordinate cobalt intramolecularly, the endogenous B_{12}–nucleotide moiety also modifies the organometallic reactivity of the B_{12} derivatives.[14,33–35,37,38]

To explore the chemical consequences of the interaction of corrinoids with a nucleotide environment in covalent models, we set out to prepare conjugates of methylcobalamin (**2**) with (deoxy)nucleotides.[33,34] Our model approach



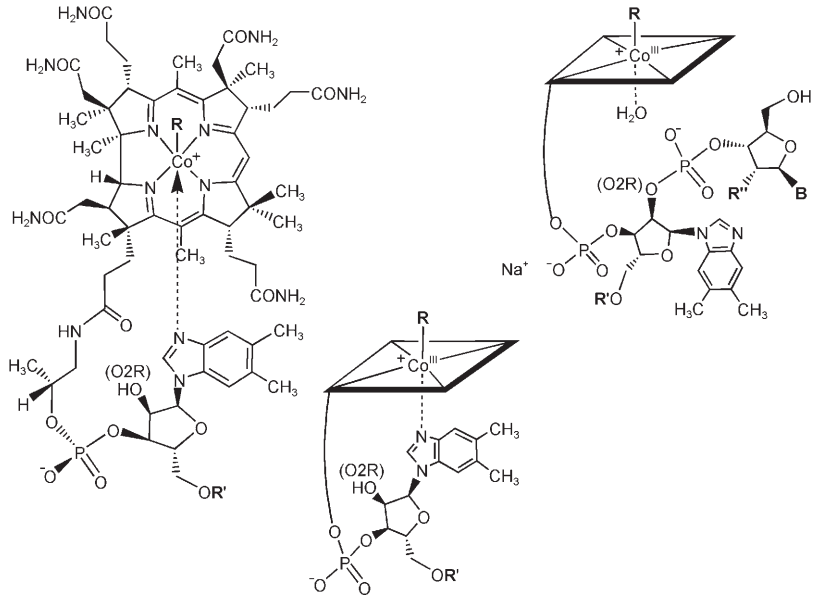
Scheme 1. B_{12} as a constitutional molecular switch. The nucleotide base of the B_{12} cofactors is coordinated to a cobalt center in the base-on form or de-coordinated in the base-off form. The two forms represent constitutional isomers and differ by the (bond) connectivity; see refs [35] and [36].

relied on the specific anchoring of nucleotides at the 2′-OH group of the ribose unit of the DMB nucleotide. The introduction of large appendages at this site was expected to exert steric effects in the cavity of base-on B_{12} derivatives and to help switch the B_{12}–nucleotide conjugates from the base-on into the base-off form (i.e., to act as B_{12}-*retro*-riboswitches).[34] As reported recently, this process was realized in a covalent deoxythymidine conjugate of **2**, Na-**4** (Scheme 2),[34] and in a related bis(deoxythymidine) conjugate of **2**, K_2-**5**.[33] As a consequence of the switch of the conjugate K_2-**5** to the base-off form, its methyl-group donor activity was enhanced.[33]

Herein, we describe the partial synthesis and spectroscopic analysis of 2′→3″-nucleotide conjugates of **2** and **3**, in which a guanosyl unit was specifically attached at the 2′-OH group of the B_{12} ribose segment and which were designed as "B_{12}-*retro*-riboswitches"[34] (Scheme 2). As derived for the deoxythymidyl appendage to methylcobalamin in Na-**4**,[34] the appended guanosyl nucleotide in the guanosyl analogue sodium Co_β–methylcobalamin-(3″→O2R)-guaninyl-3″-ate (Na-**7**) and in the corresponding coenzyme B_{12} derivative sodium Co_β–adenosylcobalamin-(3″→O2R)-guaninyl-3″-ate (Na-**6**) was expected to also assist in yielding the base-off form. The structural and spectroscopic properties of the guanosyl conjugates Na-**6** and Na-**7** were investigated to test the broader validity of our approach towards representative B_{12}-*retro*-riboswitches.[34]

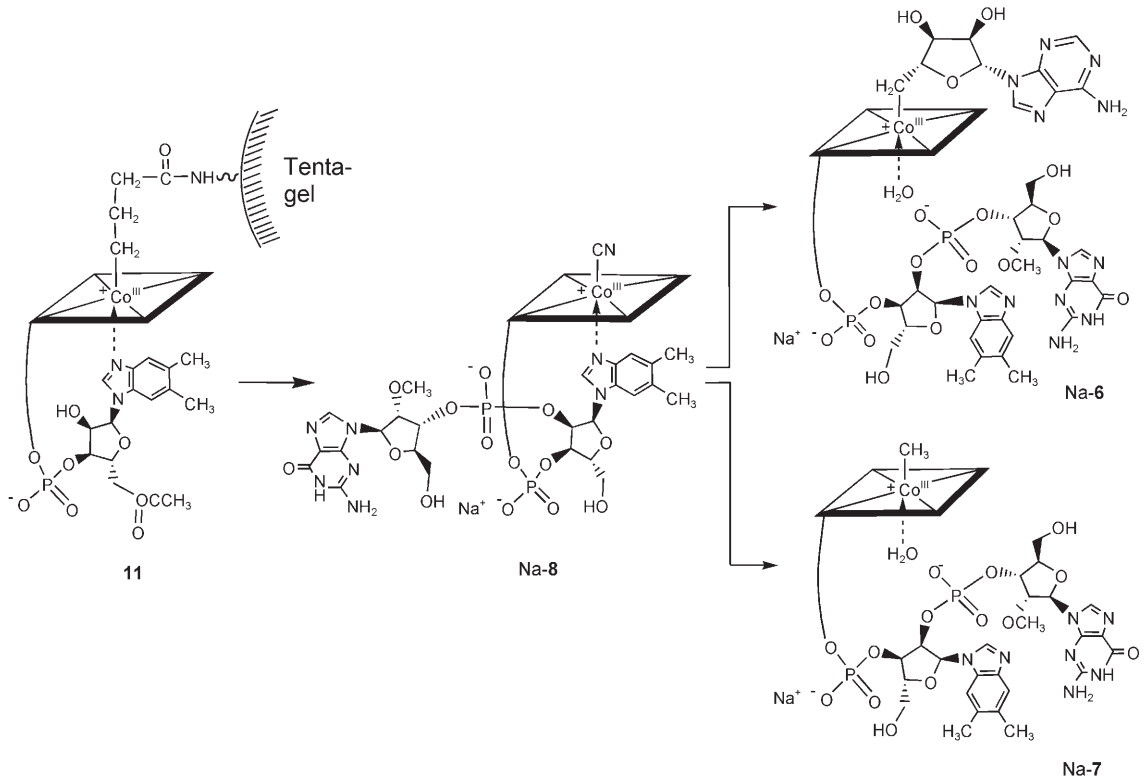
Results

The organometallic B_{12}–nucleotide conjugates Na-**6** and Na-**7** were synthesized via the cyanocorrinoid Na-**8**. This latter B_{12} derivative was prepared by using phosphoramidite chemistry[33,39] and the selective incorporation of a guanosyl unit into the B_{12} ribose unit. This approach used a solid-support strategy, in which the protected B_{12} substrate **10** was attached by its photocleavable organometallic linker on a suitable solid support.[34] An electrochemical technique[40] was applied to prepare the organometallic methyl- and deoxyadenosylcorrinoids Na-**6** and Na-**7** from the cyanocorrinoid Na-**8** (see Scheme 3 and the Experimental Section).



Scheme 2. Left: The general symbol of the base-on form and the structural formulae of vitamin B_{12} (cyanocobalamin, **1**; R = CN, R′ = H), methylcobalamin (**2**; R=CH_3, R′=H), coenzyme B_{12} (adenosylcobalamin, **3**; R=5′-adenosyl, R′=H), Co_β–cyano-O5R-acetylcobalamin (**9**; R=CN, R′=acetyl), Co_β–(3-carboxypropyl)-O5R-acetylcobalamin (**10**; R=$(CH_2)_3$–CO_2H, R′=acetyl). Right: Structural formulas of the nucleotide conjugates Na-**4** (R=CH_3, R′=acetyl, R″=H, B = thymidine), K_2-**5** (R=CH_3, R′=3′-(2′-deoxy)-thymidylate, R″=H, B = thymidine), Na-**6** (R = 5′-adenosyl, R′=H, R″=OCH_3, B = guanidine), and Na-**7** (R=CH_3, R′=H, R″=OCH_3, B = guanidine) in their base-off forms (Na-$\mathbf{4}_{off}$, K_2-$\mathbf{5}_{off}$, Na-$\mathbf{6}_{off}$, and Na-$\mathbf{7}_{off}$).

The synthesis of the modified cyanocobalamin Na-**8** was recently reported in a preliminary report: vitamin B_{12} (**1**) was protected at the 5′-OH group of the B_{12} ribose unit (O5R) as the crystalline Co_β–cyano-O5R-acetylcobalamin (**9**).[41] Co_β–(3-carboxypropyl)-O5R-acetylcobalamin (**10**) was obtained (81.5% yield) from **9** by electrochemical reduction to its Co^I form and alkylation in situ with 4-bromobutyric acid. The organometallic compound **10** was attached onto tentagel resin, which has amino group surface functionalities, to give the red solid **11** (loading was calculated to be about 143 μmol g^{-1} or 77% coverage). The tentagel-bound corrinoid **11** was coupled with 5′*O*-dimethoxytrityl-2′*O*-methyl-*N*2-(4-isopropylphenyl)oxyacetylguanosyl-3′*O*-(β-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (DMT–$mG^{iPr-PAC}$) at room temperature, with protection from humidity and light. This step was followed by a modified version of the standard oxidation/deprotection protocol, photoinduced cleavage from the solid support,[34] chromatographic



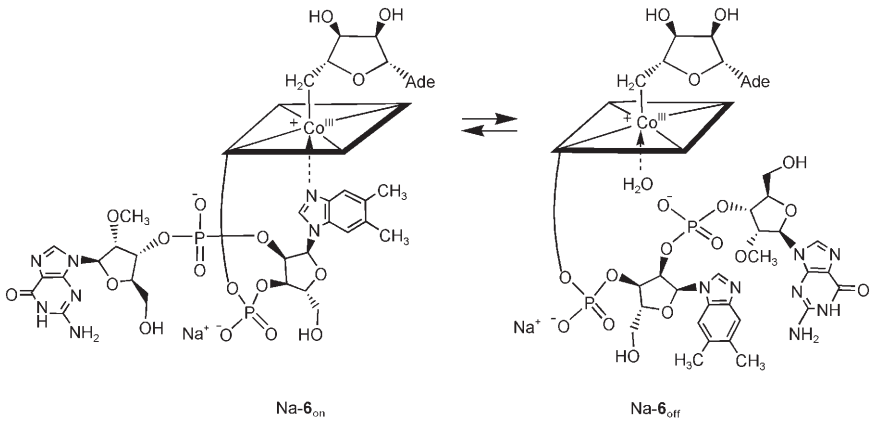
Scheme 3. Outline of the synthesis of the guanosyl B_{12} derivatives Na-**6** and Na-**7.**

purification, and precipitation to give the deprotected sodium Co_β–cyanocobalamin-(3″→O2R)-guaninyl-3″-ate (Na-**8**) in 57% yield.

For the preparation of Na-**6**, a solution of Na-**8** (4.6 mg) in methanol was electrochemically reduced to cob(I)alamin-(3″→O2R)-guaninyl-3″-ate; reaction of this compound with an excess of 5′-deoxy-5′-chloroadenosine with protection from daylight formed a cobalt–5′-adenosine species. Co_β–(5′-deoxyadenosyl)cobalamin-(3″→O2R)-guaninyl-3″-ate (Na-**6**) was obtained in 74% yield (3.9 mg) as a dark red precipitate. Compound Na-**7** was synthesized in a similar fashion by the electrochemical reduction of Na-**8** (7.5 mg, 4.3 μmol) to the corresponding cob(I)alamin-(3″→O2R)-2″-guaninyl-3″-ate, which was methylated at the cobalt center by addition of an excess of methyl iodide (with protection from daylight).[33,34] Two organometallic corrinoid compounds were obtained (according to analytical TLC) and separated by preparative reverse-phase chromatography. Sodium Co_β–methyl-O5R-acetylcobalamin-(3″→O2R)-guaninyl-3″-ate (Na-**7**) was obtained from the less polar fraction and was isolated (after precipitation from aqueous acetone) in 58% yield (4.3 mg, 2.5 μmol) as a dark red powder and was characterized as described below. The more polar fraction was obtained in 12% yield (0.92 mg, 0.52 μmol) as an orange–red precipitate (from aqueous acetone). The available spectroscopic data (see below) showed it to be the Co_α isomer of methylcorrinoid **7**, that is, Co_α–methyl-O5R-acetylcobalamin-(3″→O2R)-guaninyl-3″-ate (Na-**7**α).

Spectroscopic characterization: The suggested molecular formula of the dark red sodium Co_β–cyanocobalamin-(3″→O2R)-guaninyl-3″-ate (Na-**8**; $NaC_{74}H_{101}CoN_{19}O_{21}P_2$) was confirmed by FAB–mass-spectrometric analysis: the spectra showed pseudomolecular ions at *m/z* 1756.8 (100%, [(Na-**8**)+Na]$^+$) and *m/z* 1736.6 (52%, [(Na-**8**)+H]$^+$). The UV/Vis spectrum (and likewise the CD spectrum) of an aqueous solution of Na-**8** showed the typical features of a base-on cyanocorrinoid (e.g., see refs [42] and [43]) but exhibited an enhanced absorbance near 273 nm (a strongly positive Δε value was observed at 277 nm) as a result of the appended guanosylate group. In the NMR spectra of Na-**8** in D_2O, the signals of 72 carbon nuclei and 83 carbon-bound hydrogen atoms could be assigned (with the help of homo- and heteronuclear 2D spectra). In the ^{1}H NMR spectrum (500 MHz), the signals of the protons attached to the side-chain C32 atom ($\Delta\delta \approx -0.5$ ppm) were shielded, whereas those on the methyl group C1A and the ribose C2R atoms were deshielded ($\Delta\delta = 0.38$ and 0.7 ppm, respectively) compared to the spectrum of vitamin B_{12} (**1**)[42,43] (see the Supporting Information for atom numbering). Similarly, the ^{13}C NMR signal that arises from the C2R atom was shifted downfield by about $\Delta\delta = 5$ ppm, which is consistent with the attachment of the guanylate unit at O2R and the local-shift effects of the appended base, situated near the lower face of ring A of the corrin moiety in the base-on form of Na-**8**.

The FAB mass spectrum of the orange–red Na-**6** species confirmed its suggested molecular formula ($NaC_{83}H_{113}CoN_{23}O_{24}P_2$; see Scheme 4). A pseudomolecular ion was seen at *m/z* 1960.7 (65%, [Na-**6**+H]$^+$) and a fragment at *m/z* 1710.6 (100%, [Na-**6**+H−$C_{10}H_{12}N_5O_3$]$^+$) as a result of the loss of the organometallic group. The UV/Vis spectrum (and likewise the CD spectrum) of an aqueous solution of Na-**6** was strongly temperature dependent (as discussed further below; see also Figure 1). At room temperature, the spectra showed features typical of a base-off adenosylcorrinoid (e.g., see ref. [37]) and exhibited an enhanced absorbance near 260 nm (a strongly positive Δε value was observed at 270 nm) as a result of the appended guanosylate.



Scheme 4. Left: Na-**6** in the base-on form Na-$\mathbf{6}_{on}$. Right: Na-**6** in the base-off form Na-$\mathbf{6}_{off}$.

In the NMR spectra of Na-**6** in D_2O (measured at 26°C), the signals of 80 carbon nuclei and 91 carbon-bound hydrogen atoms could be unambiguously assigned (with the help of homo- and heteronuclear 2D spectra; see Table S2 in the Supporting Information). The well-resolved ^{1}H NMR spectrum (500 MHz) of Na-**6** was strongly temperature dependent (see Figure 2). In the spectrum taken at 26°C, the signals of a series of protons attached to the carbon atoms in the corrin, ribose, and DMB units and the organometallic adenosyl group were significantly shifted compared to **3**.[8] Most notably, protons attached to C1A ($\Delta\delta = 0.28$ ppm), C81 and C82 ($\Delta\delta = \approx 0.2$–0.7 ppm), C10 ($\Delta\delta = 0.38$ ppm), and C172 ($\Delta\delta = 0.16/0.90$ ppm) of the corrin ligand; C2R of the ribose unit ($\Delta\delta = 0.82$ ppm); and C2N ($\Delta\delta = 0.64$ ppm) and C4N ($\Delta\delta = 0.44$ ppm) of the DMB base were deshielded compared to **3**,[8] whereas those attached to C4RL ($\Delta\delta = -0.26$) and C5RL ($\Delta\delta = -0.18/-0.52$ ppm) were characteris-

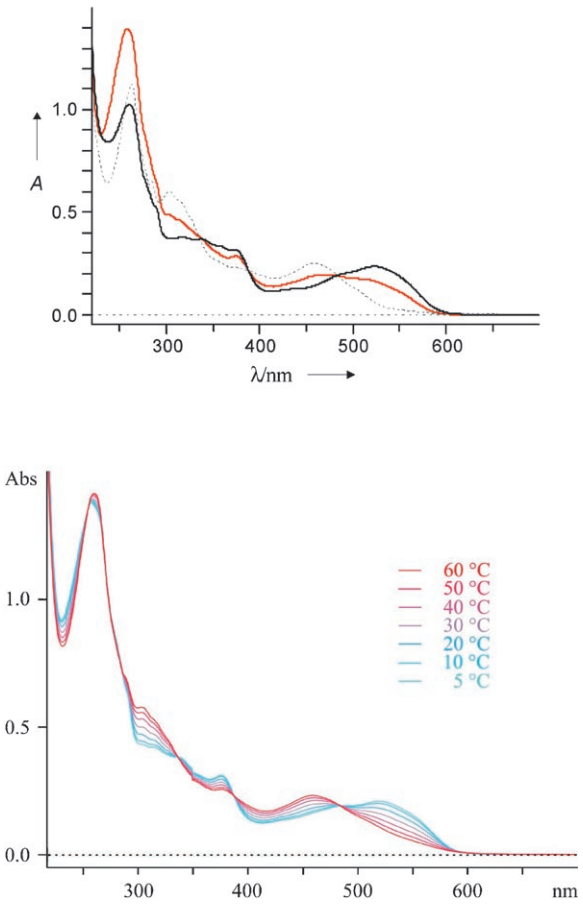


Figure 1. Top: UV/Vis spectra at 26 °C ($c = 0.00034$ M, phosphate buffer, 1 mM, pH 7.0) of Na-**6** (red, solid line), base-on **3** (black solid line, pH 7.0), and base-off **3** (dashed line, pH 1.0). Bottom: UV/Vis spectra of Na-**6** ($c = 0.00034$ M, phosphate buffer, 1 mM, pH 7.0) from 5 to 60 °C.

tically shielded (see the Supporting Information for atom numbering). Similarly, the indirectly detected ^{13}C NMR signals in the spectra of Na-**6** exhibited characteristic shifts compared to **3**.[8] The signal that arose from C2R was shifted downfield by $\Delta\delta = 4.1$ ppm, thus supporting specific attachment of the guanylate unit to O2R. However, the signals that arose from C1A were shifted downfield by $\Delta\delta = 2.1$ ppm and the signal from C5RL was shifted upfield by $\Delta\delta = 3.1$ ppm, which is consistent with a significant contribution of a base-off form of Na-**6**. Long-range ROESY cross peaks between the hydrogen atoms of the appended guanosyl unit and the corrin moiety were observed for H-C8G1 and several ribose protons on the appended nucleotide and the methyl groups C1A and C2A of the corrin ligand.

The orange-red sodium Co_β–methylcobalamin-(3″→O2R)-guaninyl-3″-ate (Na-**7**; $NaC_{74}H_{104}CoN_{18}O_{21}P_2$) was likewise confirmed by mass-spectrometric analysis to have the correct molecular formula of Na-**7** (Scheme 5): pseudomolecular ions were seen at *m/z* 1747.7 (55%, $[Na\text{-}\mathbf{7}+Na]^+$) and *m/z* 1725.4 (100%, $[Na\text{-}\mathbf{7}+H]^+$) and a strong fragment was seen at *m/z* 1710.4 (67%, $[Na\text{-}\mathbf{7}+H-CH_3]^+$). The UV/Vis spectrum (and likewise the CD spectrum) of an aqueous solution of Na-**7** was strongly temperature dependent. At room temperature, the spectra were rather similar to those of a base-on methylcorrinoid, such as methylcobalamin (**2**; e.g., see ref. [7]) and exhibited an enhanced absorbance near 263 nm (a strongly positive $\Delta\varepsilon$ value at 271 nm) as a result of the appended guanosylate group (Figure 3).

In the NMR spectra of Na-**7** in D_2O (measured at 26 °C), the signals of 69 carbon nuclei and 86 carbon-bound hydrogen atoms could be unambiguously assigned (with the help of homo- and heteronuclear 2D spectra; see the Supporting Information). In the ^1H NMR spectrum of Na-**7** (26 °C, 500 MHz), the signals of the protons attached to C1A ($\Delta\delta = 0.36$ ppm), C32 ($\Delta\delta = -0.39/-0.35$ ppm), and C172 ($\Delta\delta = -0.11/0.46$ ppm) atoms of the corrin ligand experienced the largest shift differences compared with **2**,[7,45] as was the proton attached to the ribose C2R atom ($\Delta\delta = 0.69$ ppm; see Figure 4 (top) and the Supporting Information). However, only insignificant shifts were seen at the cobalt-coordinated methyl group $Co-CH_3$ ($\Delta\delta = -0.04$ ppm) and at C2N and C4N ($\Delta\delta = 0.04$ and 0.08 ppm, respectively) of the DMB base. The similarity of the chemical-shift values associated with the signals of the hydrogen atoms attached to the methylcobalamin moieties of Na-**7** and **2**[7,45] support a structure in solution that is largely base on. Slightly shifted values of some propionic side-chain methylene protons and the methyl group C1A are indicative of altered local (de)shielding effects as a result of the appended base. ROESY spectra showed strong correlations again between the protons at C8G1, C1R1, and C5R1 of the appended nucleotide and the corrin methyl groups C1A and C2A and indicated a temporal spatial neighborhood of these groups. As expected for the Co_β-bound methyl group of Na-**7**, significant NOE interactions were observed for hydrogen atoms on the β face (at C21, C71, C12B, C17B, and C19) and at C10, C51, and C151 (in the plane) of the corrin ligand. This finding is in contrast to the NOE interactions for aqueous solutions of Na-**7**α (see below). Likewise, among the indirectly detected ^{13}C NMR signals in the spectrum of Na-**7**, only the signal that arose from C2R was shifted strongly downfield ($\Delta\delta = 5.0$ ppm, all the other shifts were shifted $\Delta\delta < 2$ ppm) compared to **2**,[45] thus supporting specific attachment of the guanylate unit at O2R.

The available spectra of the methylcorrinoid Na-**7**α helped to identify it as isomeric Co_α–methylcobalamin-(3″→O2R)-guaninyl-3″-ate (Na-**7**α). This conclusion was supported by a FAB mass spectrum, which exhibited pseudomolecular ions at *m/z* 1747.4 (66%, $[Na\text{-}\mathbf{7}\alpha+Na]^+$) and *m/z* 1725.5 (100%, $[Na\text{-}\mathbf{7}\alpha+H]^+$) and a fragment at *m/z* 1711.4 (75%). The UV/Vis spectrum of an aqueous solution of Na-**7**α at room temperature exhibited an enhanced absorbance at 262 nm as a result of the appended guanosylate species. This time, the CD spectrum exhibited a weakly positive $\Delta\varepsilon$ value at 279 nm and a strongly negative $\Delta\varepsilon$ value at 261 nm. Otherwise, these spectra were similar to the spectra of the base-off methylcorrinoids, such as Co_α–methylcobalamin[46] (Figure 3). In the ^1H NMR spectrum of Na-**7**α in D_2O (26 °C, 500 MHz; Figure 4), the signals of 85 hydrogen

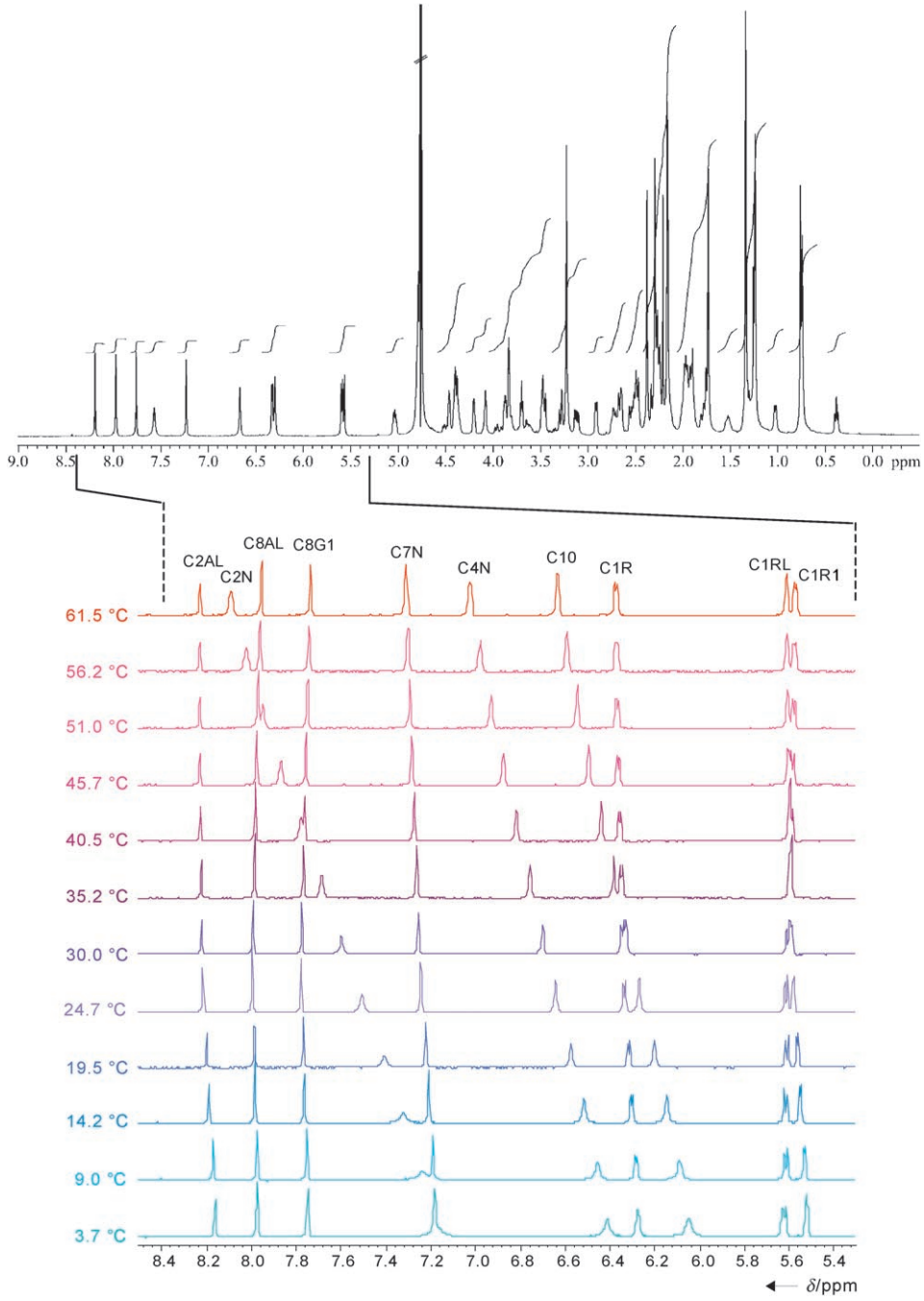
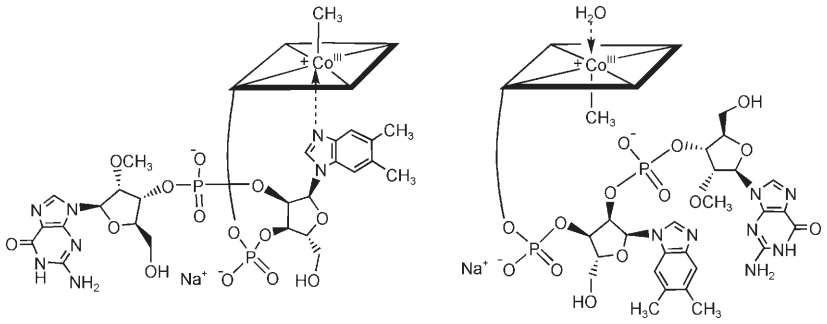


Figure 2. Top: ^{1}H NMR spectrum of Na-**6** (500 MHz, NaD_2PO_4/Na_2DPO_4 buffer, 40 mM, pD 7.07, $c = 4.5$ mM, 26 °C). Bottom: Low-field portion of the ^{1}H NMR spectra of Na-**6** at the indicated temperatures (3.7–61.5 °C, NaD_2PO_4/Na_2DPO_4 buffer, 8 mM, pD 7.07, $c = 0.94$ mM).

atoms could be identified. The high-field singlet near $\delta = -0.69$ ppm showed NOE correlations with the singlet at $\delta = 0.99$ ppm (as a result of the C1A methyl group), all of which are characteristic of the Co_α-coordinated methyl group in Na-**7**α.[47] The deduced base-off nature of Na-**7**α was also consistent with the low-field shifts of (at least) three singlets, as a result of protons attached to C10, C2N, and C4N (singlets from C7N and C8G1 that appear to remain unshifted).

UV/Vis and NMR spectroscopic analysis of the base-on/base-off equilibria of Na-6 and Na-7α: The room-temperature UV/Vis spectrum of the organometallic B_{12}–nucleotide conjugate Co_β–(5′-deoxyadenosyl)cobalamin-(3′′→O2R)-guaninyl-3′′-ate Na-**6** in aqueous solution (pH 7) featured an absorbance maximum in the visible range, which was strongly shifted hypsochromically compared to **3** (e.g., see refs [21] and [48]). The spectrum of Na-**6** exhibited an absorption maximum at 473.5 nm, thus indicating a shift of about 50 nm to shorter wavelengths compared to **3** (Figure 1). Using the absorbance coefficients at 531 nm determined for **3** (pH 7) and the protonated base-off form H-**3**$^+$ (pH 1), the spectrum of the RNA–conjugate Na-**6** was estimated to be compatible with 37% of the base-off form Na-**6**$_{off}$ and 63% of the base-on form Na-**6**$_{on}$ at 25 °C. For the base-off/base-on equilibrium of Na-**6**, the data indicate that $\Delta G_0 = -1.2$ kJ mol^{-1} at 25 °C (see Scheme 4). The base-on form Na-**6**$_{on}$ is thus stabilized to a significantly less extent in Na(**6**) than in **3**, in which the base-on form predominates strongly (K_{on} (25 °C) = 72.5[21,49]).

The UV/Vis spectra of Na-**6** were strongly temperature dependent in the investigated range of 5–60 °C (Figure 1), and with increasing temperature a steadily decreasing wavelength of the absorbance maximum in the visible region was observed. To determine the thermodynamic data and analyze the temperature-dependent UV/Vis spectra of Na-**6**, the UV/Vis spectra of Na-**6** and reference compound **3** at pH 7.0 (base-on form of **3**) and pH ≈ 1 (protonated base-off form H-**3**$^+$) were measured. The absorptions of the maximal spectral change were determined for Na-**6** and the absorption in the visible region of these organylcobalamins was used in the analysis. The extinction coefficients ε_x of Na-**6** at the selected wavelengths for the measured temperatures were calcu-



Na-**7** Na-**7**α

Scheme 5. Left: Co_β–methyl base-on form Na-**7**. Right: Co_α–methyl isomer base-off form Na-**7**α

lated. By using the temperature-corrected extinction coefficients for **3** in the base-on (ε_A) and base-off ($\varepsilon_{HA}{}^+$) forms (H-**3**$^+$), the equilibrium constant K_{on} could be determined with a two-state model [Eq. (1)].

$$K_{on} = (\varepsilon_x - \varepsilon_{HA}{}^+)/(\varepsilon_A - \varepsilon_x) \quad (1)$$

Based on the K_{on} values, the ΔG_{on} values could be calculated for the measured temperatures. To determine further the thermodynamic data, the ΔG_{on} values as a function of the temperature were used for linear regression. For Na-**6**, $\Delta H_0 = -39.1 \pm 1.8$ kJ mol^{-1} ($s = 0.41$, $N = 4$, $P = 95\,\%$) and $\Delta S_0 = -126.9 \pm 5.7$ JK^{-1} mol^{-1} ($s = 1.33$, $N = 4$, $P = 95\,\%$) were determined. For the nucleotide–conjugate Na-**6**, the data indicate a strong entropic assistance toward the formation of the base-off form, which predominates above about 35 °C. For **3**, the related extrapolation to higher temperatures indicates that the known base-on form predominates up to and beyond 100 °C (see below).[8,21,49]

The UV/Vis spectroscopic analysis of the temperature-dependent base-on/base-off equilibria of Na-**6** were supported (in a qualitative fashion) by the available 500-MHz ^{1}H NMR spectra of aqueous solutions of Na-**6** in the temperature range 3.7–61.5 °C. In these spectra (Figure 2), one set of signals (from protons on C2N, C4N, and C10) shows characteristic low-field shifts with increasing temperature. This behavior is to be expected for a shift in the organometallic corrinoid from being predominantly base-on at 3.7 °C to largely base-off at 61.5 °C (e.g., see ref. [34]). In addition, these signals sharpen, which is indicative of increasingly fast dynamics and a "fast-exchange" character of the spectra.

The UV/Vis spectrum of an aqueous solution of Na-**7** at room temperature featured an absorbance maximum in the visible range at 517 nm, which was similar to that of the thymidine analogue Na-**4** (maximum at 515 nm)[34] and shifted hypsochromically only by about 12 nm compared to **2**.[48] For both methylcorrinoids Na-**7** and Na-**4**, the UV/Vis spectra indicated a predominance of the base-on forms at room temperature. As described earlier,[34] the spectra of Na-**4** were further analyzed with respect to their temperature dependence, thus indicating a switch to the base-off form ($\Delta H_0 = -41.4 \pm 0.3$ kJ mol^{-1} ($s = 0.13$, $N = 7$, $P = 95\,\%$) and $\Delta S_0 = -117.5 \pm 1.0$ kJ^{-1} mol^{-1} ($s = 0.40$, $N = 7$, $P = 95\,\%$). In a related way, the ^{1}H NMR spectra of Na-**4** also showed a pronounced and characteristic temperature dependence in the temperature range 3.7–61.5 °C. In these spectra (Figure 5), one set of signals (from protons on C2N, C4N, and C10) shows characteristic low-field shifts with increasing temperature. Such shifts were also observed for the adenosylcorrinoid Na-**6** and are indicative of an increased population of the base-off form at higher temperatures (see above and Figure 2). However, in the ^{1}H NMR spectra of Na-**4**, the protons on C6T1 and C1R1 of the deoxythymidylate appendage showed an opposite shift to higher field with in-

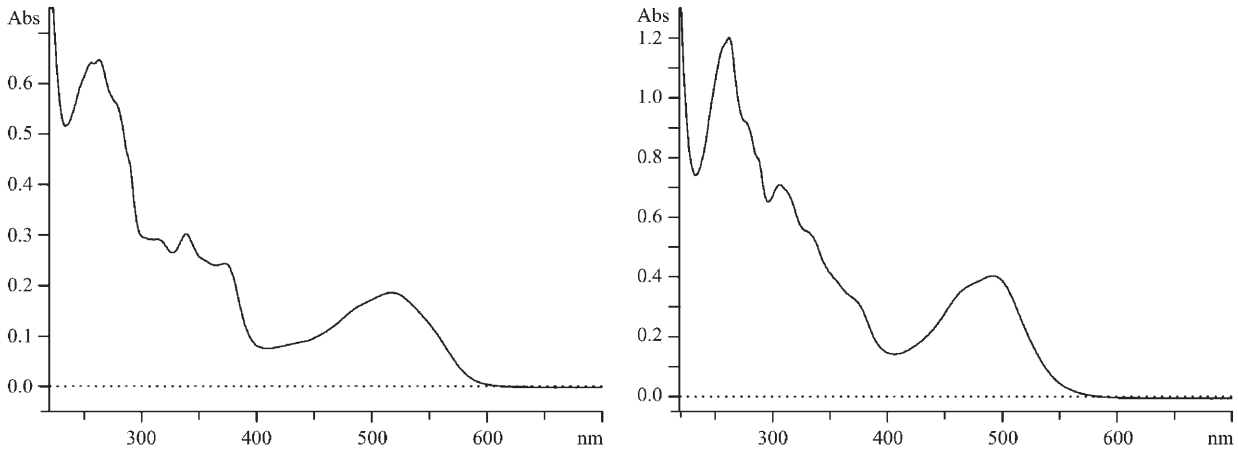


Figure 3. Left: UV/Vis spectra of Na-**7** (H_2O, $c = 2.50 \times 10^{-4}$ M). Right: UV/Vis spectra of Na-**7**α (H_2O, $c = 4.58 \times 10^{-4}$ M).

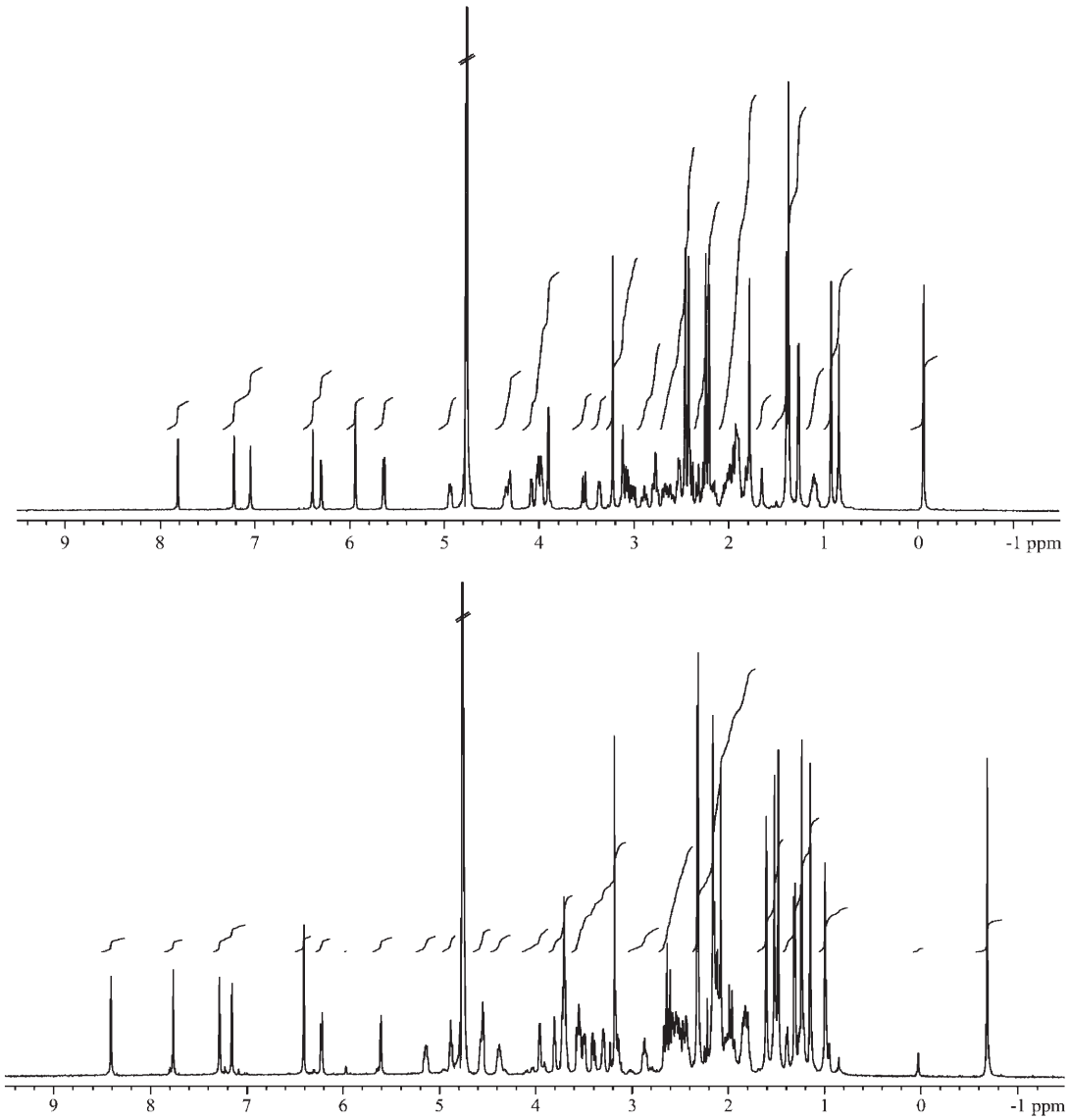


Figure 4. Top: ^{1}H NMR spectrum of Na-**7** (D_2O, $c = 1.7$ mM, 26 °C, 500 MHz). Bottom: ^{1}H NMR spectrum of Na-**7**α (D_2O, $c = 1.7$ mM, 26 °C, 500 MHz).

creasing temperature (see Figure 5), which is possibly an indicator of a (more effective hydrophobic) interaction with the DMB base in the base-off form.

Discussion

B_{12} riboswitches versus B_{12}-*retro*-riboswitches: In B_{12}-dependent enzymes, B_{12} cofactors have been observed to be bound in the base-on or base-off forms.[35,50] For enzyme-bound methylcorrinoids, this observation has been rationalized in the direct context of their role as methyl group-transfer catalysts.[13,35] On one side, the enzyme may control the reactivity of the bound corrinoid cofactor by alternatively binding it either in the base-off/his-on or completely base-off forms.[10,51] In a complementary fashion, "complete" methyl corrinoids may also be considered to help reorganize ("switch") the protein environment through the ability to coordinate a histidine residue in the base-off form (this behavior is believed to be particularly relevant in multimodular B_{12}-dependent enzymes, such as methionine synthase).[10,51] In an artificially evolved B_{12}-binding RNA species (the "B_{12} aptamer"),[29] binding of vitamin B_{12} was characterized by crystallographic studies and was found to occur in the base-on form (however, all major interactions with the aptamer involved the β face of the bound corrinoid).[32] In the newly discovered B_{12} riboswitches, the binding of coenzyme B_{12} (**3**) causes a conformational switch of the RNA, thus controlling further expression of the corresponding gene.[25,26] The mode of B_{12} binding to B_{12} riboswitches apparently was not addressed in the earlier study.[25,26] Recent spectroscopic work indicated base-on binding of **3**

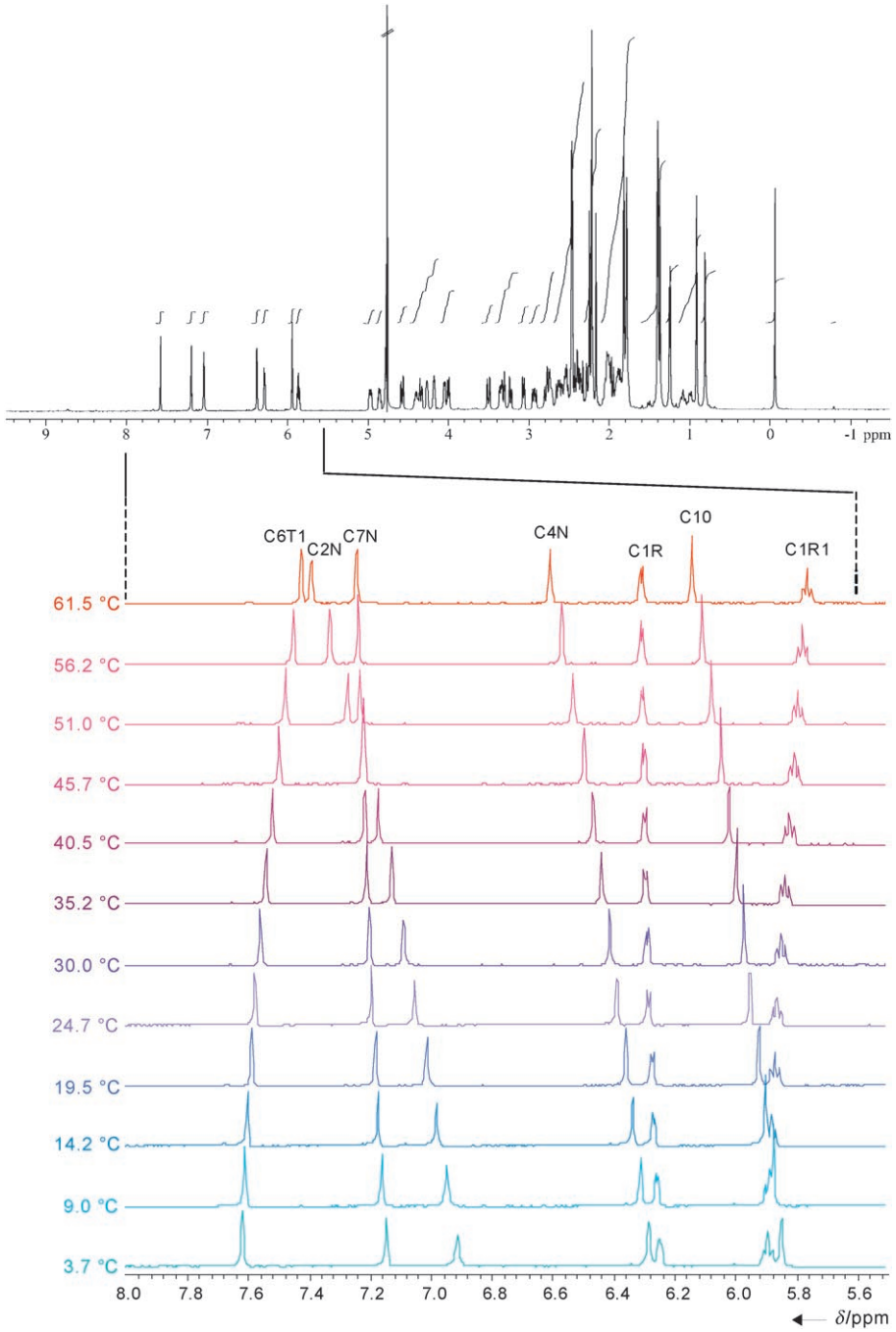


Figure 5. Top: ^{1}H NMR spectrum of Na-**4** (D_2O, $c = 2.0$ mM, 26 °C, 500 MHz). Bottom: Low-field section of the ^{1}H NMR spectra of Na-**4** at the indicated temperatures (D_2O, $c = 0.9$ mM, 3.7–61.5 °C, 500 MHz).

by the B_{12} riboswitch in the 5′-untranslated region of btuB-RNA from *E. coli*,[52] which codes for the B_{12}-transporting btuB protein in this microorganism.[20]

The existence of complete B_{12} derivatives, such as **1**–**3**, in one of the isomeric base-on or base-off forms is the consequence of the unique capacity of complete corrinoids to coordinate their appended nucleotide moiety reversibly to the (redox-active) cobalt center and—thereby—to behave as constitutional molecular switches[34] (e.g., see refs [36] and [53]). In solution, the equilibrium of this intramolecular coordination of the DMB nucleotide function is effectively controlled by external factors, such as the acidity of the solution and redox level, which may control the mutual interaction of the DMB base and the cobalt center. In proteins, external ligands (histidine) and a binding interface for the nucleotide moiety are the major contributors to the formation of the base-off/his-on form.[33,34] Alternatively, interactions with (oligo)nucleotides (i.e., DNA or RNA) may possibly also induce the base-on/base-off switch of the B_{12} moiety of complete corrinoids. This notion was proposed and examined in earlier exploratory investigations with DNA conjugates of methylcobalamin[33,34] and is now extended herein.

In our model approach, attached nucleotide moieties are to be tested for their possible ability to switch the delicate coordinative base-on/base-off equilibrium[35,54] and to provide (in this way) precedence for B_{12}-*retro*-riboswitches.[34] This approach relies on the specific introduction of nucleotide appendages, which are expected to exert significant steric effects in the cavity of base-on B_{12} derivatives, at the 2′-OH group of the ribose unit of the DMB nucleotide. Such a steric factor would be expected to (help to) switch the B_{12}–nucleotide conjugates from the base-on to the base-off form.

Spectral characterization of base-on to base-off switching: The room-temperature UV/Vis absorbance spectrum of a pH neutral aqueous solution of Na-**6** indicated a significant shift of this complete organometallic corrinoid toward the base-off form. The absorption maximum in the spectrum of Na-**6** occurred at 473 nm and was shifted hypsochromically by more than 50 nm compared with **3**. The base-on form of Na-**6** at 25 °C was more stable by merely $\Delta G_o = 1.28$ kJ mol^{-1} than its base-off form. Under similar experimental conditions, the corresponding thermodynamic value for **3** at 25 °C was determined earlier as $\Delta G_0 = -10.7$ kJ mol^{-1}.[49,54]

The UV/Vis spectrum of the organometallic RNA conjugate Na-**6** is pronouncedly temperature dependent (Figure 1, bottom). This finding is another noteworthy contrast to the situation with **3**, for which temperature had hardly any effect on the UV/Vis absorbance spectra. The absorbance maximum at long wavelengths in the spectrum of Na-**6** shifts from near 520 nm at low temperature (predominantly base-on form) to 459 nm at elevated temperatures (Figure 1, bottom), which is indicative of a switch to a predominantly base-off form. By using (temperature-corrected) extinction coefficients of the UV/Vis spectra of **3** in aqueous solution at pH 7 and H-**3**$^+$ at pH 1, the spectra of Na-**6** could be fitted (in a two-state model) for the amounts of the base-on and base-off forms, so that $\Delta H_o = -39(\pm 2)$ kJ mol^{-1} and $\Delta S_0 = -127(\pm 6)$ JK^{-1} mol^{-1} could be estimated. When compared to the data for **3** ($\Delta H_0 = -23.4(\pm 4)$ kJ mol^{-1} and $\Delta S_0 = -54.3(\pm 12)$ JK^{-1} mol^{-1}[54]), it is indicated that the enthalpy and entropy values are therefore both more negative in the RNA conjugate Na-**6**. However, the entropic contribution is more than twice as large and more effective (near ambient temperature) in Na-**6**, and the temperature dependence of the base-on to base-off switch is much more pronounced in the RNA conjugate Na-**6**. Accordingly, for Na-**6** the constant for the base-off to base-on equilibrium K_{on}(Na-**6**) is strongly temperature dependent and decreases from about $K_{on} = 1.7$ at 25 °C to $K_{on} = 0.5$ at 50 °C. The base-on form at 25 °C is disfavored by about 45-fold compared to the corresponding equilibrium of **3** (K_{on}(25 °C) = 77).[54] The latter value indicates that the appended guanosine base in Na-**6** causes a shift of approximately $\Delta G_0 = 9$ kJ mol^{-1} (25 °C) toward the base-off form Na-**6**$_{off}$. A similar behavior was observed earlier with Co$_\beta$–methyl-O5R-acetylcobalamin-(3″→O2R)-2″-deoxythymidyl-3″-ate (Na-**4**).[34] The thymidine appendage of Na-**4** was found to also cause a remarkably similar shift ($\Delta G_0 = 9$ kJ mol^{-1}) toward the base-off form Na-**4**$_{off}$ in this DNA conjugate of methylcobalamin.[34]

In the course of the isolation experiments, the B$_{12}$–nucleotide Na-**4** was deacetylated to the DNA conjugate Na-**4d**, which crystallized in the base-on form Na-**4d**$_{on}$.[34] The cobalamin moiety of Na-**4d**$_{on}$ had similar geometric characteristics to **2**. However, a sodium counterion was bound in a remarkable bidentate, bridging fashion to OP1R (of the phosphate group attaching the 2″-deoxythymidyl-3′-ate to O2R of the B$_{12}$ ribose unit) and to OPR (of the phosphate group of the B$_{12}$–nucleotide loop), thus forming a nine-membered ring including a bound sodium ion. The well-structured, base-on form Na-**4d**$_{on}$ found in the crystal is compatible with the NMR studies of Na-**4**. At room temperature and in aqueous solution, it is indicated by the NMR data that Na-**4** also predominantly exists as a structure with similar characteristics to **2** (with respect to the cobalamin moiety).

The base-on forms of B$_{12}$ derivatives in solution may also be induced by protonation to de-coordinate their DMB nucleotide and reversibly switch into a (protonated) base-off form.[55] This acid-induced constitutional switch can easily be observed by a color change and a large shift in the UV/Vis absorbance maxima (e.g., Figure 1, top). The basicity of the complete corrinoids and the pK_a values of their protonated base-off forms thus indicate the acidity of the solution when the switch is induced and are a measure of the stabilization by formation of the (deprotonated) base-on form. From analysis of the UV/Vis and CD spectra of Na(**4**) at various pH values of the solution, pK_a(H-**4**) = 3.78(±0.02) was determined.[34] The pK_a(H-**4**) value thus was higher by about 0.88 than for (H-**2**)$^+$,[54] indicating that the **4**$^-$ ion is about six times more basic and has a less strongly coordinated DMB base than **2**.[34]

Consistent with the observation of the same magnitude of the thermodynamic effects of the thymidine and guanosine appendages on the base-on/base-off equilibria, the UV/Vis and ^{1}H NMR spectra at room temperature of the DNA conjugate Na-**4** and RNA conjugate Na-**7** (the methyl analogue of Na-**6**) exhibited similar characteristics. However, in contrast to the 5′-deoxyadenosyl derivative Na-**6**, for which (as expected) only one coordinative isomer was found, in the isolation of Co$_\beta$–methylcorrinoid Na-**7**, the Co$_\alpha$ isomer Na-**7**α was also present and could be isolated and characterized spectroscopically (see Scheme 5). The isomeric pair Na-**7**/Na-**7**α illustrates the existence of the rare α isomers of cobalamins. Such α isomers have typically been observed in "incomplete" corrinoids,[21,47,48] whereas these isomers have been observed in complete methylcorrinoids in cases in which the nucleotide coordination was weak or inhibited by protonation of the nucleotide base.[21,33,48] The ^{1}H NMR and UV/Vis spectra of Na-**7**α showed the characteristic signatures of the organometallic Co$_\alpha$ isomers[47] and, therefore, of the base-off forms (Figure 3 and Figure 4).[33]

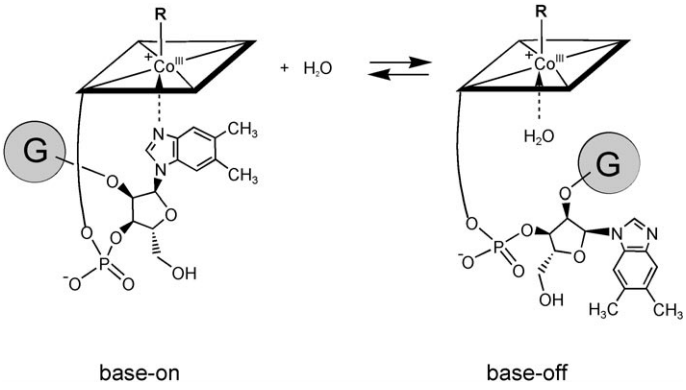
The observed weak DMB coordination in Na-**7** opens up access to two unusual coordination patterns: the base-off Co$_\beta$–methyl form Na-**7**$_{off}$ and the Co$_\alpha$–methyl coordination isomer Na-**7**α.[47] This situation is in contrast to **2**, in which the by far dominant structure features a Co$_\beta$-bound methyl group and allows strong *trans*-axial Co$_\alpha$ coordination of the DMB base. These results illustrate the relevance of the DMB coordination on the stability of organometallic B$_{12}$ derivatives, as discussed earlier for **2** and the related methylcorrinoids.[36,56]

In B$_{12}$-dependent enzymes, the B$_{12}$ coenzymes have been observed to be bound either in the base-on or base-off forms.[11–17,57–59] A B$_{12}$-binding antibody[60] and human B$_{12}$-binding proteins[61] induced the base-off to base-on switch of the bound B$_{12}$ derivatives. These two latter cases illustrate the scope of the protein environment to induce a constitutional switch of complete B$_{12}$ cofactors that is opposite to that observed in some well-known B$_{12}$-dependent enzymes.[10] Indeed, the resulting change of the structure of bound organometallic B$_{12}$ cofactors typically also changes their reactivity as catalysts in chemical and enzymatic reactions.[36]

As shown herein, an RNA environment may impose similar structural changes on a bound B$_{12}$ cofactor and thus influence the organometallic reactivity of the corrinoid. Our interest in this novel aspect of B$_{12}$ chemistry[33,34] was strengthened by the recent discovery of the relevance of

direct interactions between B_{12} derivatives and an RNA environment in B_{12} riboswitches.[25,27,62,63] This latter finding established the direct interaction between RNA and B_{12} coenzymes to represent a new mechanism of genetic control, as suspected in related genetic experiments.[64]

In contrast to the studies of the B_{12} riboswitches, in which mRNA was observed to restructure as the result of B_{12} binding,[25,26] our investigation focuses on the reverse effect in the B_{12} moieties. In the organometallic B_{12}–nucleotides Na-**4**, Na-**6**, and Na-**7**, the attached nucleotide moieties induce the base-on form to reconstitute to the base-off form (Scheme 6). The B_{12}–nucleotide conjugates Na-**4**, Na-**6**, and



Scheme 6. B_{12}–guanosyl conjugates as molecular switches. The appended guanosyl unit (G) assists in the constitutional switch of B_{12} cofactors from the base-on form (with a coordinated endogenous DMB base) to the (de-coordinated DMB) base-off form.

Na-**7** represent covalent models of B_{12}-*retro*-riboswitches, as it is their cobalamin moieties that undergo a coordinative switch under the influence of the appended (deoxy)nucleotide.[34] This constitutional switch turned out to be strongly temperature dependent and driven by large entropic effects. "*Retro* switching" by a nucleotide environment may also accompany B_{12} binding to B_{12} riboswitches, as the complete corrinoids themselves can be switched between the base-on and base-off forms.

Corrinoids are unique biological organometallic catalysts[35] and are essential in basic metabolic processes in archea and in a range of other microorganisms, even encompassing (marine) algae.[65] A variety of anaerobes that do not have the biosynthetic capacity to build up DMB cobamides (the cobalamins, such as **1**–**3**) depend on alternative complete corrinoids, such as adeninylcobamides.[66] The pattern of nucleotide bases observed in natural complete B_{12} cofactors[35,66,67] points at a remarkable tolerance and biosynthetic incorporation of a range of RNA-derived heterocycles.[68] A complete base-off form with a β-guanosylate appendage is a notable conserved stage in the biosynthesis of the cobamides.[3,69] By eventual replacement of the β nucleotide moiety by the unique α nucleotides of the various cobamides, they are processed further[3] to incorporate the apparent structural prerequisite for the base-on forms of the complete corrinoids, as typical of cobalamins and adeninylcobamides.[4,35]

The basic structural elements of coenzymes, in general,[70] and of the corrinoids, in particular,[4] have been suggested to be of pre-enzymatic origin. Coenzyme B_{12} (or a simpler molecular progenitor) may, for example, have played a crucial role in the presumed original appearance of DNA through the reduction of ribonucleotides.[71] The existence and reactivity of corrinoids is thus of particular interest in the context of the hypothesis of an early RNA-based form of life.[72] However, complete corrinoids not only have evolved first (of all) to be remarkable (organometallic) cofactors, but also to be unique biological molecular switches. The concept of *retro*-riboswitching[34] explores the chemical basis for inducing a coordinative base-on to base-off switch in B_{12}–nucleotide conjugates and is largely complementary to the phenomenon relevant in mRNA species that bind coenzyme B_{12} (B_{12} riboswitches),[25,26] in which the direct interaction between B_{12} derivatives and RNA restructures the RNA species.[73] This latter finding[73] highlights the discovery of the importance of direct metabolite binding to RNA as a means of genetic control. On the other hand, the question of how catalytic functions could have arisen in a controlled way in a nucleotide environment has also been frequently asked in more recent times.[74,75] When addressing this issue, the incorporation of natural cofactors into artificial ribozymes has been considered recently.[76–78] We have proposed that corrinoids are also included in such investigations.[33,34] Clearly, the exceptional organometallic reactivities of B_{12} derivatives, which are modified by the action of their own (natural and endogenous) nucleotide function,[35] invite further studies with designed or evolved nucleotide environments to obtain a better view on the hypothetical roles of B_{12} cofactors in pre-enzymatic life processes.

Experimental Section

General: Water was purified by Epure, Barnstead Co.; acetone, CH_2Cl_2 (filtered over basic aluminum oxide of activity grade 1), MeOH, Hg, tetra-*n*-butylammonium hexafluorophosphate (TBAHFP), MeI, AcOH, KCl, KCN, NaH_2PO_4, Na_2HPO_4, iodine, sym-collidine, and aq. HCl solution were obtained from Fluka (puriss) or Fluka MicroSelect; sodium 3-(trimethylsilyl)propane sulfonate (TSP) and THF (absolute, stored over molecular sieves ($H_2O \leq 0.005\,\%$) were obtained from Fluka (puriss); MeCN, benzoic acid, methylamine solution (41 % in water), methylamine solution (ca. 33 % in absolute ethanol), and phenoxyacetic anhydride (Pac_2O) were obtained from Fluka, (purum); 1-methylimidazole was obtained from Fluka (puriss) for DNA synthesis; sodium perchlorate monohydrate was obtained from Fluka (purum, cryst.); 5′-chloro-5′-deoxyadenosin (for preparation see ref. [79]); MeCN (gradient grade for liquid chromatography) was obtained from Merck; 1,2-dichloroethane (extra pure) was obtained from Riedel-de Haën; dichloroacetic acid was obtained from Riedel-de Haën; 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-methyl-N^2-[{(4-isopropylphenyl)oxy}acetyl]guanosine-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite was obtained from Glen Research Corporation; benzylthiotetrazole (BTT) was prepared according to ref. [80]; reverse-phase chromatographic materials were obtained from Merck; aluminum oxide was obtained from Sigma; Sep-Pak C_{18} cartridges was obtained from Waters. pH values were measured with a WTW SenTix 41 electrode connected to a WTW inoLab digital pH meter.

Electrosynthetic experiments: O_2-sensitive reactions were performed in a glove box (Mecaplex GB-80, <10 ppm O_2). The electrochemical syntheses[40] were carried out in an electrolysis cell with two compartments separated by a medium-porosity glass frit and a Hg-pool working electrode, Pt-wire counter electrode, 0.1 N calomel electrode (CE) as a reference electrode (potentiostat Amel 550). The reaction work up was carried out outside of the glove box in a dark room under dim light.

Automated oligonucleotide synthesis: A Pharmacia Gene Assembler Special was used for solid-phase oligonucleotide synthesis, with a modified protocol provided by Pharmacia. Moreover, the support was allowed to swell in dry acetonitrile for 1 h prior to the synthesis and the cartridge with the support was shaken during the coupling step (15-min coupling). Acetonitrile was dried over molecular sieves (4 Å). Phosphoramidite (40 mg) in dry acetonitrile (400 μL) was used for each coupling; 0.35 M solution of BTT in dry acetonitrile was added for activation; detritylation solution: 4% of dichloroacetic acid in 1,2-dichloroethane; oxidation solution: acetonitrile (50 mL), iodine (200 mg), sym-collidine (4.6 mL), and water (23 mL); capping: 0.2 M Pac_2O in THF (solution A) and 0.4 M methylimidazole and 0.2 M sym-collidine in THF (solution B).

Spectral measurements: UV/Vis spectra (λ_{max} [nm], $\log\varepsilon$ [$dm^3mol^{-1}cm^{-1}$]) were recorded on Hitachi U-3000 apparatus. CD spectra ($\lambda_{max/min}$ [nm], θ [$10\,deg\,cm^2mol^{-1}$]) were recorded on a Jasco J-715 spectropolarimeter. NMR spectra: were recorded on a Varian Unity 500 plus spectrometer; ^1H NMR (500 MHz) in H_2O/D_2O 9:1 or D_2O (δ(HDO) = 4.76 ppm), ^{13}C NMR (125 MHz) in H_2O/D_2O 9:1 or D_2O (external standard δ(TSP) = 0 ppm), ^{15}N NMR (51 MHz) in H_2O/D_2O 9:1 (external standard $\delta(NH_{3(l)}) = 0$ ppm), ^{31}P NMR (202 MHz) in D_2O (external standard δ(phosphoric acid, 85%) = 0 ppm); apparent coupling constants are given as J values [Hz]; spectra were recorded at 26 °C unless mentioned otherwise. FAB-MS spectra (*m/z* (relative intensity)) were recorded on a Finnigan MAT 95S spectrometer with a nitrobenzyl alcohol (NOBA) matrix and Cs^+ bombardment (for atom numbering, see ref. [44] and the Supporting Information).

Syntheses and spectral analyses: Co_β–cyano-O5R-acetylcobalamin (**9**), Co_β–(3-carboxypropyl)-O5R-acetylcobalamin (**10**), tentagel-immobilized O5R-acetylcobalamin (**11**), sodium Co_β–methyl-O5R-acetylcobalamin-(3″→O2R)-thymidylate (Na-**4**), and sodium Co_β–cyano-O5R-acetylcobalamin-(3″→O2R)-thymidylate (Na-**12**) were prepared as described.[34]

Sodium Co_β–cyano-O5R-acetylcobalamin-(3″→O2R)-thymidylate (Na-12):[34] For the ^1H and ^{13}C NMR spectra (H_2O/D_2O, 9:1), see data in Table S1 in the Supporting Information; ^{15}N NMR (51 MHz, H_2O/D_2O 9:1): δ = 110.0 (N84), 110.5 (N34), 112.1 (N134), 114.7 (N183), 117.7 (N73), 118.8 (N23), 124.1 ppm (N174); ^{31}P NMR (202 MHz, D_2O): δ = −1.67 (P1, correlates to H-C3R1, H-C2R, H-C1R in ^1H,^{31}P HMBC spectrum), −0.45 ppm (P, correlates to H_3-C177, H_b-C175, H-C176, H-C3R in ^1H,^{31}P HMBC spectrum).

Sodium Co_β–cyanocobalamin-(3″→O2R)-2″-*O*-methylguanylate (Na-8): (For the preliminary results, see ref. [34].) Tentagel-immobilized O5R-acetylcobalamin (**11**; 54.22 mg with ρ = 132 μmol g^{-1} and 13.01 mg with ρ = 144 μmol g^{-1}) and B_{12} (9.03 μmol) were put into the synthesis cartridge for automated oligonucleotide synthesis. After the preparative steps were carried out by the machine, the cartridge was removed and dried under high vacuum. In the dark room, a support with crude protected tentagel-bound B_{12}–mononucleotide was added to a solution (degassed by bubbling with argon for several minutes) of methylamine in water (2 mL) and methylamine in ethanol (2 mL) in a centrifuge tube and the suspension was stirred at room temperature for 2 h under argon. The solution was removed by centrifugation and the red solid was washed with methanol/water 1:1 (2×) and methanol (5×) and dried overnight under high vacuum. The red solid was then suspended in water (4 mL) and stirred for swelling of the tentagel for 2 h in the dark. The well-stirred suspension was cooled in an ice bath and irradiated for 30 min with a 500-W tungsten lamp at a distance of approximately 20 cm. The red solution was separated from the support by centrifugation. The support was washed with water and the photolysis process was repeated twice. The red solutions containing corrinoids were combined and the amount of corrinoids was determined to be 7.37 μmol by UV/Vis spectroscopic analysis (absorption at 354 nm, ε = 22 700 $dm^3mol^{-1}cm^{-1}$). The raw, orange-red aquo–B_{12} conjugate (in 9.5 mL of water) was treated with a 0.1 M solution of KCN/HOAc 1:1 (147 μL, 14.7 μmol) and stirred at room temperature under protection from light for 40 h (conversion into the Co_β–cyano forms). The solvents were removed from the dark-red solution and the residue was redissolved in a minimum amount of aqueous 80 μM sodium perchlorate solution. Crude red Na-**8** was precipitated by addition of acetone and purified by column chromatography on RP-18 (16.1 g) with an acetonitrile/water eluent (gradient: 5–7% acetonitrile in steps of 1%, 30 mL of each; 8% acetonitrile: 60 mL). The fraction with Na-**8** was analyzed by UV/Vis spectroscopic analysis (absorption at 359.5 nm, ε = 25 000 $dm^3mol^{-1}cm^{-1}$) and dried. The red residue was dissolved in water containing sodium perchlorate (50 μmol) and was precipitated with acetone to yield 8.97 mg (57.2%) of powdery Na-**8**. R_f = 0.79 (RP-18, MeCN/H_2O 3:7); for the ^1H and ^{13}C NMR data (D_2O), see data in Table S1 in the Supporting Information; UV/Vis (H_2O, c = 3.1×10^{-4} M): λ_{max} ($\log\varepsilon$) = 546.5 (3.89), 359.5 (4.41), 273.5 nm (4.35 $dm^3mol^{-1}cm^{-1}$); CD (H_2O, c = 3.1×10^{-4} M): $\lambda_{max/min}$ (θ) = 544.5 (−5200), 492.5 (−10700), 432.0 (37 000), 362.0 (−64 300), 325.0 (−15 300), 308.5 (−13 400), 277.5 (27 400), 249.5 nm (−40 300 ($10°\,cm^2mol^{-1}$); MS (FAB, NOBA): *m/z* (%): 1761.6 (23), 1760.6 (36), 1759.6 (93), 1758.6 (100) $[(Na\text{-}\mathbf{8})+Na]^+$; 1757.6 (20), 1756.6 (21), 1738.5 (28), 1737.8 (41), 1736.6 (52) $[(Na\text{-}\mathbf{8})+H]^+$ ($NaC_{74}H_{102}CoN_{19}O_{21}P_2$); 1734.6 (20), 1733.6 (38), 1732.5 (38) $[(Na\text{-}\mathbf{8})+Na-CN]^+$; 1731.6 (20), 1730.7 (31), 1710.6 (20) $[(Na\text{-}\mathbf{8})+H-CN]^+$; 1708.6 (21).

Sodium Co_β–(5′-deoxyadenosyl)cobalamin-(3″→O2R)-2″-*O*-methylguanylate (Na-6): See also ref. [34]: In a glove box, Na-**8** (4.6 mg, 2.6 μmol) and benzoic acid (0.78 mg, 6.4 μmol) were dissolved in the electrolyte solution (5 mL; 0.1 M TBAHFP in methanol) and placed into the cathodic compartment of the electrolysis cell. The red solution was stirred magnetically and reduced at −1.1 V versus 0.1 N CE reference electrode (at room temperature) for 3 h. To the remaining green solution, containing only Co^I (as determined by UV/Vis spectroscopic analysis), 5′-chloro-5′-deoxyadenosine (7.6 mg, 26.6 μmol) was added under protection from light. Stirring was continued for 9.5 h, while the cathode was kept at the same potential. The solution was transferred into a dark room, diluted with water (20 mL), treated with sodium perchlorate (11.2 mg), and extracted with dichloromethane (4×30–40 mL each). The orange-red aqueous phase was evaporated, the residue taken up in a minimum amount of water, treated with sodium perchlorate (6 mg, 16 equiv), and precipitated by the addition of acetone. The crude product was purified by column chromatography on RP-18 (4.0 g) with an acetonitrile/water eluent (gradient: 5–12% acetonitrile in steps of 1%, 10 mL of each; 13% acetonitrile: 25 mL). The nucleotide conjugate **6** eluted at 11–13% acetonitrile as two broad fractions that were not separated (probably different salts). Precipitation from water/acetone in the presence of sodium perchlorate (3.7 mg, 10 equiv) yielded Na-**6** (3.88 mg; 74.7%). R_f = 0.51 (RP-18, MeCN/H_2O 3:7, 20 mM $NaClO_4$); for the ^1H and ^{13}C NMR data (NaD_2PO_4/Na_2DPO_4 buffer, 40 mM, pD 7.07) see data in Table S2 in the Supporting Information; UV/Vis (NaH_2PO_4/Na_2HPO_4 buffer, 1 mM, pH 7.0, c = 3.4×10^{-4} M): λ_{max} ($\log\varepsilon$) = 473.5 (3.76), 374.5 (3.92), 257.0 nm (4.61 $dm^3mol^{-1}cm^{-1}$): see Figure 1 for the UV/Vis spectra at different temperatures; CD (NaH_2PO_4/Na_2HPO_4 buffer, 1 mM, pH 7.0, c = 3.4×10^{-4} M): $\lambda_{max/min}$ (θ) = 550.0 (−11 800), 479.5 (17 300), 432.0 (−10 700), 391.5 (8600), 360.5 (−16 800), 333.0 (21 200), 302.5 (9600), 288.0 (−3000), 269.5 (20 600), 240.0 nm (−24 000 [$10°\,cm^2mol^{-1}$]); MS (FAB, NOBA): *m/z* (%): 1983.5 (23), 1962.6 (29), 1961.7 (68), 1960.7 (65) $[(Na\text{-}\mathbf{6})+H]^+$ ($Na\times C_{83}H_{114}CoN_{23}O_{24}P_2$); 1938.8 (23) $[(Na\text{-}\mathbf{6})-Na+2H]^+$; 1734.6 (25), 1733.5 (44), 1732.5 (45) $[(Na\text{-}\mathbf{6})+Na-Ado(C_{10}H_{12}N_5O_3)]^+$; 1713.5 (25), 1712.6 (57), 1711.5 (75), 1710.6 (100) $[(Na\text{-}\mathbf{6})+H-Ado(C_{10}H_{12}N_5O_3)]^+$; 1709.6 (38), 1708.7 (35), 1689.6 (25).

Sodium Co_β–methylcobalamin-(3″→O2R)-2″-*O*-methylguanylate (Na-7): In a glove box, Na-**8** (7.5 mg, 4.3 μmol) and benzoic acid (1.3 mg, 10.6 μmol) were dissolved in the electrolyte solution (5 mL; 0.1 M TBAHFP in methanol) and placed into the cathodic compartment of the electrolysis cell. The red solution was stirred magnetically and reduced at −1.1 V versus 0.1 N CE reference (at room temperature). After 2.5 h, 0.87 C (Q_t = 0.83 C) had been consumed and methyl iodide (0.8 μL, 1.8 mg, 12.7 μmol) was added to the green solution under protection from light. The solution was reduced at the same potential for 1.5 h and

then transferred into a dark room. The solution was diluted with water (15 mL), treated with sodium perchlorate (10.5 mg), and extracted with dichloromethane (4×30 mL each). The red aqueous phase was filtered over dry cotton wool and evaporated, the residue was taken up in a minimum amount of water and treated with sodium perchlorate (5.5 mg, 45 μmol). Acetone was added dropwise, until a slight turbidity appeared. After storage in the refrigerator overnight (no crystals were yielded), the crude product was first desalted and then purified by column chromatography on RP-18 (4.0 g, desalting: water (40 mL), chromatography with an acetonitrile/water eluent (gradient: 2–22% acetonitrile in steps of 2%, 10 mL of each)). Two main fractions were collected (fraction 1: 14–18% acetonitrile, fraction 2: 20–22% acetonitrile), which had the same R_f value (RP-18, MeCN/H_2O 3:7). According to the ^{1}H NMR spectra, fraction 1 was a mixture of α- and β-methylated mononucleotides and fraction 2 contained only the β-methylated product Na-**7**. Fraction 1 was again subjected to column chromatography on RP-18 (4.0 g). Elution with acetonitrile/20 mM aqueous $NaClO_4$ (gradient: 5–12% acetonitrile in steps of 1%, 10 mL of each; 13–16% acetonitrile in steps of 1%, 20 mL of each; 17–20% acetonitrile in steps of 1%, 10 mL of each) gave fractions I (elution at 15–16% acetonitrile) and II (eluent: 17–20% acetonitrile), which were collected and desalted with Sep-Pak C_{18} cartridges (general procedure: MeOH (20 mL); H_2O (20 mL); loading with H_2O or buffer; desalting with H_2O (30–40 mL); elution with H_2O/MeOH 1:1 (5 mL) and MeOH (5 mL)). The UV/Vis spectra indicated that fraction I was the base-off Co_α–methyl–Co(III)–corrin (Na-**7**α) and fraction II was the Co_β–methyl isomer Na-**7**. The solvents of both fractions were removed on a rotary evaporator. The residues were taken up in water, treated with sodium perchlorate (ca. 10 equiv), and precipitated by the addition of acetone to yield Na-**7**α (0.92 mg; 12.3% of fraction I) and Na-**7** (4.32 mg; 58.0% of fraction II).

Na-7: R_f=0.34 (RP-18, MeCN/H_2O 3:7, 20 mM $NaClO_4$); for the ^{1}H and ^{13}C NMR data (D_2O): see Table S3 in the Supporting Information; UV/Vis (H_2O, $c=2.50\times10^{-4}$ M): λ_{max} ($\log\varepsilon$)=517.5 (3.87), 372.0 (3.99), 339.0 (4.08), 313.0 (4.07), 263.0 nm (4.41 $dm^3mol^{-1}cm^{-1}$); CD (H_2O, $c=2.50\times10^{-4}$ M): $\lambda_{max/min}$ (θ)=557.0 (−8400), 486.5 (34600), 428.0 (−11300), 385.0 (13700), 355.5 (−27000), 321.0 (−21700), 271.0 (17600), 254.0 nm (−29100 [10°cm^2mol^{-1}]; MS (FAB, NOBA): *m/z* (%): 1748.4 (60), 1747.7 (55) $[(Na\text{-}7)+Na]^+$, 1733.5 (42), 1732.5 (36) $[(Na\text{-}7)+Na-CH_3]^+$, 1726.5 (66), 1725.4 (100) $[(Na\text{-}7)+H]^+$ ($NaC_{74}H_{105}CoN_{18}O_{21}P_2$), 1711.6 (86), 1710.4 (67) $[(Na\text{-}7)+H-CH_3]^+$.

Sodium Co_β–methyl-O5R-acetylcobalamin-(3″→O2R) thymidylate (Na-4):[34] For the ^{1}H and ^{13}C NMR data (H_2O/D_2O 9:1), see Table S3 in the Supporting Information; ^{15}N NMR (51 MHz, H_2O/D_2O 9:1): δ=110.0 (N84), 110.7 (N34), 112.7 (N134), 114.4 (N183), 117.6 (N73), 118.9 (N23), 123.0 ppm (N174); ^{31}P NMR (202 MHz, D_2O): δ=−1.61 (P1, correlates to H-C3R1, H-C2R in a ^{1}H,^{31}P HMBC spectrum), −0.44 ppm (P, correlates to H_b-C175, H-C176, H-C3R in a ^{1}H,^{31}P HMBC spectrum).

Sodium Co_α–methylcobalamin-(3″→O2R)-2″-*O*-methylguanylate (Na-7α): Obtained with approximately 5% Na-**7** as an impurity. R_f=0.40 (RP-18, MeCN/H_2O 3:7, 20 mM $NaClO_4$); ^{1}H NMR (D_2O): δ=−0.69 (s, 3H), 0.99 (s, 3H), 1.15 (s, 3H), 1.22–1.27 (m), overlapped with 1.23 (s), total 4H; 1.31 (d, *J*=8 Hz), 1.47–1.53 (m), overlapped with 1.48 (s) and 1.52 (s), total 7H; 1.61 (s, 3H), 1.82 (m, 3H), 1.93–2.18 (m), overlapped with 2.08 (s) and 2.16 (s), total 15H; 2.31 (s, 3H), 2.32 (s, 3H), 2.42–2.67 (m, 8H), 2.86 (m, 1H), 3.13–3.58 (m), overlapped with 3.18 (s), total 9H; 3.70 (m, 3H), 3.80 (m, 1H), 3.95 (m, 1H), 4.38 (m, 1H), 4.55 (m, 2H), 4.88 (m, 1H), 5.14 (m, 1H), 5.60 (d, *J*=2.4 Hz, 1H), 6.22 (d, *J*=4.2 Hz, 1H), 6.40 (s, 1H), 7.15 (s, 1H), 7.28 (s, 1H), 7.76 (s, 1H), 8.41 ppm (s, 1H); UV/Vis (H_2O, $c=4.58\times10^{-4}$ M): λ_{max} ($\log\varepsilon$)=492.0 (3.95), 306.5 (4.19), 262.0 nm (4.42 $dm^3mol^{-1}cm^{-1}$); CD (H_2O, $c=4.58\times10^{-4}$ M): $\lambda_{max/min}$ (θ)=506.0 (42700), 455.5 (−22200), 381.5 (10400), 352.0 (−9200), 329.5 (19300), 293.5 (−1800), 279.0 (1200), 260.5 nm (−48200 [10°cm^2mol^{-1}]); MS (FAB, NOBA): *m/z* (%): 1747.4 (66) $[(Na\text{-}7)\alpha)+Na]^+$, 1727.4 (53), 1726.4 (54), 1725.5 (100) $(Na\text{-}7)\alpha)+H]^+$ ($NaC_{74}H_{105}CoN_{18}O_{21}P_2$), 1711.4 (75).

Analysis of the ^{1}H NMR spectra of Na(4) and Na(6) as a function of temperature: ^{1}H NMR spectra of Na-**4** (c=0.9 mM, D_2O, 500 MHz) and Na-**6** (c=0.94 mM, D_2O/8 mM NaD_2PO_4/Na_2DPO_4, pD 7.07) were recorded in the range 3.7–61.5°C; the chemical-shift data of diagnostic proton signals were analyzed as a function of temperature (see Figure 2 and Figure 5).

Analysis of the UV/Vis spectra of Na-4 and Na-6 as a function of temperature: To determine the thermodynamic data for Na-**4** and Na-**6**, the temperature-dependent UV/Vis spectra of these compounds and the reference compounds methylcobalamin (**2**) and coenzyme B_{12} (**3**) each in the base-on and protonated base-off forms were analyzed. The wavelengths of the maximal spectral change were determined for Na-**4** and Na-**6** and the wavelength in the β region of the spectra of the alkylcobalamins was used. The extinction coefficients ε_x of Na-**4** and Na-**6** at the selected wavelengths for the measured temperatures were calculated. By using the temperature-corrected extinction coefficients for **2** and **3** in the base-on (ε_A) and base-off (ε_{HA}) forms, the equilibrium constant K_{on} could be determined with a two-state model [see Eq. (1)].

Based on the K_{on} values, ΔG_{on} could be calculated for the data at each temperature (see Table 1 and Table 2). The ΔG_{on} values were obtained as a function of temperature and were used to determine, from a linear regression, the values of ΔH_0 (from the intercept) and ΔS_0 (from the slope). The following thermodynamic values were obtained: for Na-**4**: $\Delta H_0=-41.4\pm0.3$ kJmol^{-1} (s=0.13, N=7, P=95%); $\Delta S_0=-117.5\pm1.0$ J$K^{-1}mol^{-1}$ (s=0.40, N=7, P=95%); for Na-**6**: $\Delta H_0=-39.1\pm1.8$ kJmol^{-1} (s=0.41, N=4, P=95%) and $\Delta S_0=-126.9\pm5.7$ JK^{-1} mol^{-1} (s=1.33, N=4, P=95%).

Table 1. Thermodynamic data of Na-**4**[a] from temperature-dependent UV/Vis spectra.[b]

T	**2**	H-**2**$^+$		Na-**4**		
[K]	ε	ε	Abs.	ε	K_{on}	ΔG_{on} [kJmol^{-1}]
293	7840	2130	0.375	7590	22.2	−7.55
303	7820	2070	0.361	7300	10.1	−5.81
313	7750	2000	0.342	6920	5.92	−4.63
323	7670	1970	0.318	6440	3.62	−3.46
333	7560	1940	0.290	5870	2.31	−2.32
343	7440	1950	0.258	5230	1.48	−1.13
353	7290	1960	0.227	4500	0.98	0.06
363	7110	1950	0.198	4000	0.66	1.25

[a] Na-**4**: $\Delta H_0=(-41.4\pm0.3)$ kJmol^{-1}; $\Delta S_0=(-117.5\pm1.0)$ J$K^{-1}mol^{-1}$ (for details, see the text). [b] Conditions: temperature range: 20–90°C; extinction coefficients ε of **2**, H-**2**$^+$, and Na-**4** at 528 nm; for Na-**4** ($c=4.9\times10^{-4}$ M) and **2** ($c=5.0\times10^{-4}$ M): NaH_2PO_4/Na_2HPO_4 buffer, 10 mM, pH 7.0; for H-**2**$^+$ ($c=5.0\times10^{-4}$ M): 0.1 M HCl, pH 1.

Table 2. Thermodynamic data of Na-**6**[a] from temperature-dependent UV/Vis spectra.[b]

T	**3**	H-**3**$^+$		Na-**6**		
[K]	ε	ε	Abs.	ε	K_{on}	ΔG_{on} [kJmol^{-1}]
293	6570	1380	0.168	4940	2.19	−1.91
303	6430	1380	0.144	4250	1.31	−0.69
313	6270	1390	0.121	3550	0.80	0.60
323	6080	1430	0.101	2970	0.49	1.89

[a] Na-**6**: $\Delta H_0=(-39.1\pm1.8)$ kJmol^{-1}; $\Delta S_0=(-126.9\pm5.7)$ J$K^{-1}mol^{-1}$ (for details, see the text). [b] Conditions: temperature range: 20–50°C; $c=3.4\times10^{-4}$ M, extinction coefficients ε of **3**, H-**3**$^+$, and Na-**6** at 531 nm; for Na-**6** and **3**: NaH_2PO_4/Na_2HPO_4 buffer, 1 mM, pH 7.0; H-**3**$^+$: 0.1 M HCl, pH 1.

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