Synthesis of Enantiomerically-Pure [^{13}C]Aristeromycylcobalamin and Its Reactivity in Dioldehydratase, Glyceroldehydratase, Ethanolamine Ammonia-Lyase and Methylmalonyl-CoA Mutase Reactions

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Abstract: We describe a novel enantioselective synthesis of aristeromycin, the carbocyclic analogue of adenosine. The seven-step synthesis is also suitable for the preparation of specifically-labelled [6′-^{13}C]aristeromycin. Both the unlabelled and ^{13}C-labelled product was coupled to vitamin B_{12} to form aristeromycylcobalamin. This carbocyclic analogue of coenzyme B_{12} was examined for its coenzymic activity with several adenosylcobalamin-dependent enzymes. For glyceroldehydratase and dioldehydratase, the reaction rate (k_{cat}) was 38 and 44% of that measured with adenosylcobalamin as coenzyme. In contrast, aristeromycylcobalamin showed no detectable activity with methylmalonyl-CoA mutase and ethanolamine ammonia-lyase. Instead, it was a weak inhibitor of the former and a strong inhibitor of the latter enzyme. The slower turnover rate with glyceroldehydratase raised the hope of detecting the 6′-deoxyaristeromycyl radical intermediate. Comparison of the EPR spectra of the intermediates in the glyceroldehydratase reaction, which used adenosyl- and aristeromycylcobalamines, respectively, as coenzyme, revealed a significant shift and this suggests a different geometric position of these cofactors at the binding site during the cleavage of the carbon-cobalt bond. However, we found no evidence for the existence of a 6′-deoxyaristeromycyl radical during the reaction with [6′-^{13}C]aristeromycylcobalamin. We conclude that the lifetime of this radical is still too short to be observed.

Keywords: cobalamines · cofactors · enzyme catalysis · EPR spectroscopy · kinetics · synthetic methods

Introduction

Adenosylcobalamin (AdoCbl) is an essential cofactor of various enzymes that catalyse rearrangement reactions.[1–4] A unique property of AdoCbl, which is also known as coenzyme B_{12}, is the covalent cobalt-carbon bond. This bond is stable in aqueous solution in the dark but undergoes homolysis when exposed to light.[5] In the enzyme-bound state this homolysis is reversible, strictly controlled, and is the starting step in the rearrangement reactions. The cleavage of the cobalt–carbon bond generates the highly reactive 5′-deoxyadenosyl radical, which is able to abstract a hydrogen atom from a non-activated position of the substrate. The substrate radical is equally unstable but is protected from possible reaction partners so that it can rearrange cleanly to the product radical. Hydrogen atom transfer to the product radical from the 5′-methyl group of the intermediate 5′-deoxyadenosine is followed by regeneration of AdoCbl to complete the catalytic cycle.

The corresponding enzymes are able to accelerate the homolysis of the cobalt–carbon bond by a factor of up to 10^{12},[6, 7] but only on substrate binding.[8–10] Obviously, a portion of the binding energy is used to induce a conformational change which weakens the cobalt–carbon bond. The occurrence of radical intermediates can be directly shown by EPR spectroscopy. When more than one such species are present, only the more stable species, that is those that have the longest lifetime, can be detected.

Upon homolysis of the cobalt – carbon bond, the relatively stable paramagnetic cob(II)alamin and the extremely unstable deoxyadenosyl radical are formed. Thusfar the deoxyadenosyl radical has not been observed spectroscopically, but its intermediacy has been documented by numerous experiments with deuterium and tritium-labels.[11, 12] In adenosylcobalamin-dependent enzymes, rather stable organic radicals, which are generated by hydrogen abstraction by the deoxyadenosyl radical from the substrate or product, have been detected by EPR spectroscopy.[8, 13] The localisation of the carbon-centred radicals in the substrate/product has been experimentally

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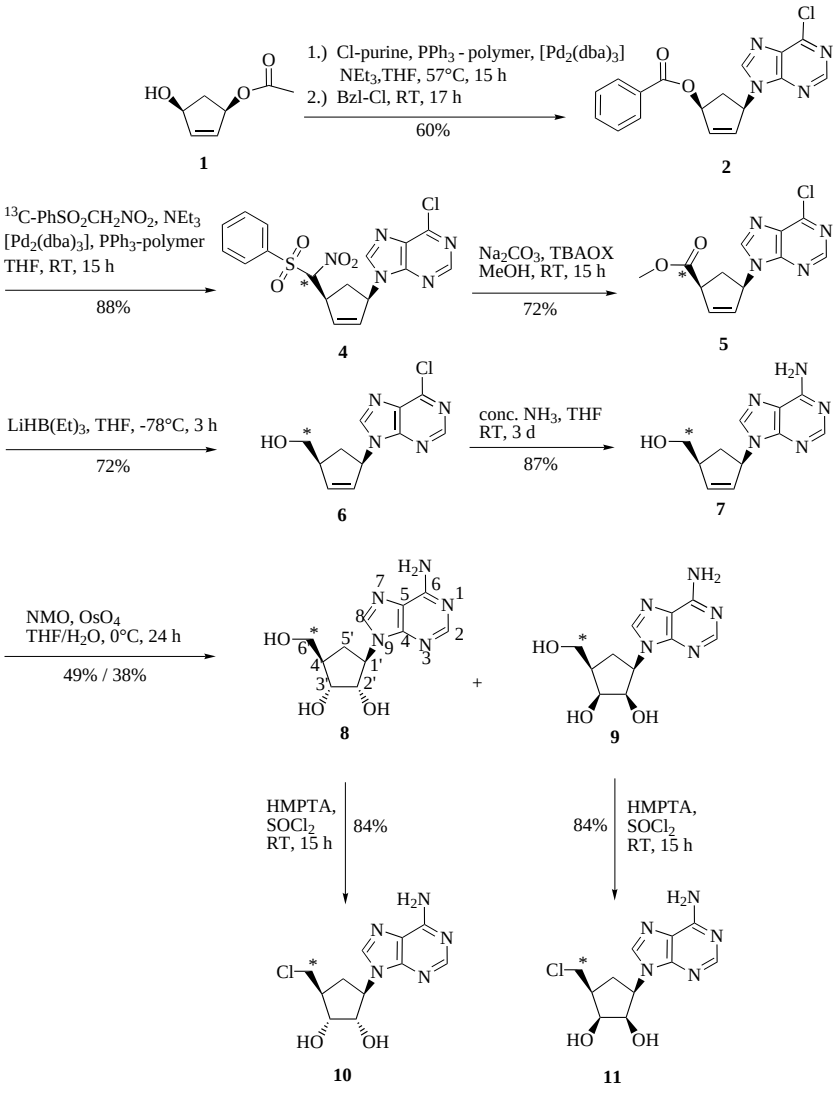
determined for ethanolamine ammonia-lyase,[14] glutamate mutase,[15] and 2-methyleneglutarate mutase (A. J. Pierik, unpublished results) by EPR spectroscopy of the enzyme in the presence of isotopically-labelled substrates. These studies and the rapid-freeze experiments performed by Babior et al.[14] and Leutbecher et al.[16] exclude substantial concentrations of amino acid-centred and deoxyadenosyl radical species as intermediates.

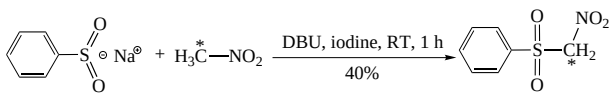
Attempts to detect the 5′-deoxyadenosyl radical have involved its stabilisation by the introduction of a double bond into the ribose ring. The use of 3′,4′-anhydroadenosylcobalamin as a cofactor gave rise to the more stable allylic 3′,4′-anhydroadenosyl radical. This analogue lacks the 3′-OH group and was only slightly active with B_{12}-dependent enzymes. Its oxygen sensitivity is very high and this led to its decomposition in a few minutes.[17] Abeles and co-workers have prepared an analogue of coenzyme B_{12}, in which instead of the adenosyl group, racemic aristeromycin was covalently bound to the central cobalt.[18] The coenzymic activity of this carbocyclic analogue in the dioldehydratase reaction was about 30% of that measured with AdoCbl. It was concluded that replacement of the ribosyl O-atom by methylene does not severely affect the reaction or, in other words, that this oxygen atom does not play an essential role in the catalytic process. We succeeded in achieving a novel enantioselective synthesis of aristeromycin and its [6′-^{13}C] isotopomer for the investigation of the coenzymic activity of aristeromycylcobalamin with several B_{12}-dependent enzymes. After the stereoisomerically-pure carbanucleoside was coupled to cobalamin, the coenzyme analogue was used for kinetic and EPR measurements.



Scheme 1. Synthesis of 6′-^{13}C-labelled aristeromycin (**8**) and its 6′-chlorinated deoxy-analogue (**10**).

Results and Discussion

Synthesis of (2′*S*,3′*R*,4′*R*)-(−)-aristeromycin (**8**), its 2′,3′-bis-epi isomer (**9**) and its [6′-^{13}C]-isotopomer (Scheme 1): The starting material of this synthesis was the commercially-available and enantiomerically-pure (1*S*,4*R*)-*cis*-acetoxycyclopent-2-ene-1-ol (**1**) which was coupled to chloropurine by the method of Trost et al.[19] The reaction was carried out in the presence of trisdibenzylidene acetone dipalladium(0) which was converted in situ into the catalytically-active but oxygen-sensitive tetrakistriphenylphosphine palladium(0). We used a commercially available polymer-bound triphenylphosphine instead of free triphenylphosphine. Although this required a longer reaction time at 57 °C, this avoided a tedious removal of the side product, triphenylphosphine oxide. After the polymer-bound material was removed by filtration, the intermediate allylic alcohol was reacted with benzoylchloride to give (1′*R*,4′*S*)-4′-benzyloxy-1′-(6-chloro-9*H*-purin-9-yl)cyclopent-2′-ene (**2**) in 60% yield. Since an excess of triethylamine was present in solution from the previous step, HCl, which had been simultaneously generated, was immediately neutralized. In the next step, the benzoate group was substituted by the hydroxymethyl moiety. Here we could introduce the ^{13}C-label. The anion formed from phenylsulphonylnitromethane (**3**) was most suitable for this purpose. Compound **3** was prepared by the method of Wade et al.[20] but with major modifications (Scheme 2). As nitromethane is commercially available with a [^{13}C]enrichment $\geq$99%, it was treated with sodium phenylsulphinate in the presence of



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Scheme 2. Synthesis of phenylsulphonyl[^{13}C]nitromethane (**3**) from sodium phenyl-sulphinate and [^{13}C]-labelled nitromethane.

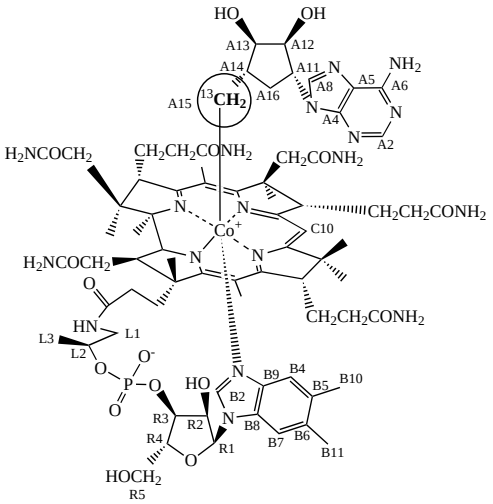
iodine and the base 1,8-diazobicyclo[5.4.0]undec-7-ene (DBU) to give phenylsulphonylnitromethane (**3**) in 40% yield. The substitution of the benzoate group of **2** was again accomplished by the method of Trost et al.[19] using the same reagents as previously described for the step **1** → **2**; however, this reaction took place at room temperature. Triethylamine served both to start the catalytic cycle and to deprotonate **3** to form the corresponding carbanion. Nitrosulphonate **4** was obtained in 88% yield as a nearly 1:1 mixture of diastereomers (with the introduction of a new chiral centre!). Separation of the diastereomers was unnecessary as oxidative cleavage of nitrosulphonate **4** with tetrabutylammonium oxone (TBAOX) in MeOH/CH_2Cl_2 gave the methylester **5** with the concomitant abolition of the asymmetric centre that was created in the previous step.[19] The last two steps also offer the possibility of specifically introducing an isotope label into other carbocyclic nucleosides such as carbovir or abacavir, which are important antiviral agents.[21–26]

Reduction of methylester **5** by "superhydride" (lithium triethylborohydride) at −78 °C afforded the alcohol **6** in 72% yield. The use of stronger reducing agents could attack the chloropurine moiety. In fact, even with "superhydride", the reaction mixture had to be quenched with ethylacetate before it was allowed to warm to room temperature. The chloro-substituent of the purine ring was then replaced with an amine group by stirring **6** in aqueous ammonia at room temperature. The resulting carbocyclic nucleoside **7** was easily purified and gave colourless crystals in 87% yield. Finally *cis*-hydroxylation of **7** using osmium tetroxide in catalytic amounts afforded aristeromycin (**8**) and its 2′,3′-bis-epimer **9**. The reaction was carried out at 0 °C in the presence of excess methylmorpholine-*N*-oxide and gave **8** as the major product (49%) and its 2′,3′-bis-epimer **9** (38%). Compounds **8** and **9** were separated by HPLC.

The synthesis of [6′-^{13}C]-(−)-aristeromycin ([6′-^{13}C](**8**)) was achieved from the monoacetate **1** in seven steps with an overall yield of 12%.

Synthesis of aristeromycylcobalamin (12), and 2′,3′-bis-*epi* aristeromycylcobalamin (13): Aristeromycin (**8**) and its 2′,3′-bis-*epi* isomer (**9**) were converted into their 6′-chloro-6′-deoxy-analogues **10** and **11** as previously described.[27] Thionylchloride in hexamethylphosphoramide was used as chlorination agent. In this way, only the primary hydroxyl group was substituted. The products were purified by ion exchange chromatography.

In the last step of the synthesis, hydroxycobalamin was reduced with sodium borohydride in presence of cobalt(II)-chloride to cob(I)alamin (vitamin B_{12s}).[12] Cob(I)alamin was then alkylated with the 6′-chloro-6′-deoxyaristeromycins (**10**) and (**11**) to afford aristeromycylcobalamin (**12**), and its 2′,3′-bis-*epi* analogue (**13**) in 60 and 55% yield, respectively (Scheme 3). Due to the high light sensitivity of the alkylcobalamins, both of the last reaction steps and the subsequent purification by HPLC was carried out in the dark. Compounds **12** and **13** and their [6′-^{13}C]isotopomers were characterized by NMR and mass spectrometry.



Scheme 3. [6′-^{13}C]aristeromycylcobalamin (**12**) and its 2′,3′-bis-epi isomer (**13**). The NMR signals were annotated with the following letters: **A**: aristeromycyl, **B**: benzimidazole, **C**: corrin, **L**: loop, **R**: ribosyl. Position of the 2′- and 3′-hydroxy groups of the aristeromycyl moiety: **12**: *anti*, **13**: *syn*.

Kinetic investigations: Aristeromycylcobalamin served as coenzyme for diol- and glyceroldehydratase. K_M values were identical within experimental error to those of adenosylcobalamin for both these enzymes. However, their k_{cat} values were reduced to 44 and 38% of the values for dioldehydratase and glyceroldehydratase, respectively. Considering the high amino-acid sequence identity of the two enzymes, their similar acceptance of aristeromycylcobalamin was not surprising. Despite their high sequence identity, glyceroldehydratase has a much higher affinity for both coenzymes (Table 1). It seems that the ether oxygen of the ribosyl moiety in AdoCbl does not play a role in the mechanism of the reaction. The somewhat reduced k_{cat} values may arise from the slightly different geometry of the cyclopentane moiety with respect to the furanose ring.[28]

Table 1. Kinetic properties of coenzyme B_{12} and aristeromycylcobalamin with glycerol-dehydratase (*C. freundii*) and dioldehydratase (*S. typhimurium*).

Compound	Glyceroldehydratase		Dioldehydratase	
	K_M [nM]	k_{cat} [10^{-3} s^{-1}]	K_M [μM]	k_{cat} [10^{-3}s^{-1}]
adenosylcobalamin	10 ± 1	173 ± 3	0.54 ± 0.06	233 ± 8
aristeromycylcobalamin	11 ± 2	65 ± 1	0.57 ± 0.03	102 ± 1

Surprisingly, methylmalonyl-CoA mutase and ethanolamine ammonia-lyase seemed unable to use aristeromycylcobalamin as coenzyme. While the mutase was only weakly inhibited, ethanolamine ammonia-lyase was strongly inhibited by the coenzyme analogue (Table 2). In our kinetic investigations we used the "parallel" method, that is the simultaneous addition of AdoCbl and aristeromycylcobala-

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Table 2. Kinetic properties of coenzyme B_{12} and aristeromycylcobalamin with methylmalonyl-CoA mutase (*P. shermanii*) and ethanolamine ammonia-lyase (*S. typhimurium*).

Compound	Methylmalonyl-CoA mutase		Ethanolamine ammonia-lyase	
	K_m [nM]	K_i [nM]	K_m [nM]	K_i [nM]
adenosylcobalamin	57 ± 4	–	65 ± 5	–
aristeromycylcobalamin	–	830 ± 45	–	16 ± 2

min.[29] The experimental data were analyzed by Dixon linearization. For ethanolamine ammonia-lyase details for determination of the apparent K_i value are shown in Figure 1. The K_i value for the lyase is four times lower than the value of K_M for AdoCbl and is comparable to that for hydroxycobalamin ($K_i = 12.7$ nM[30]).

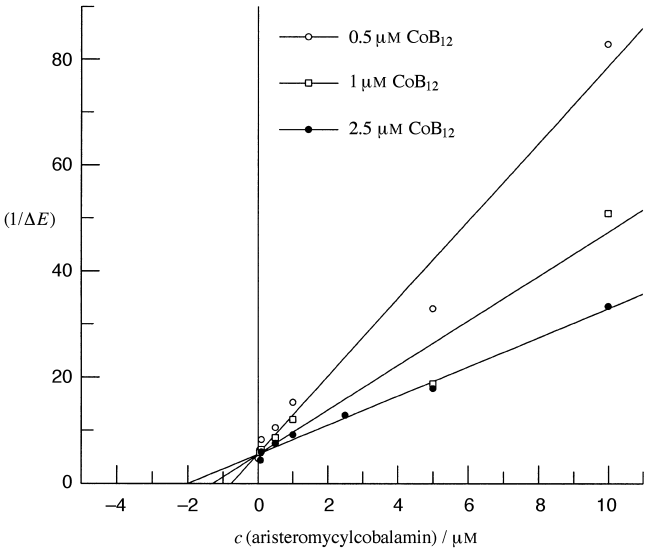

Figure 1. Dixon linearization obtained by the "parallel" addition method for determination of the K_i value of aristeromycylcobalamin with ethanolamine ammonia-lyase (*S. typhimurium*). Rate measurements were started with synchronous addition of coenzyme B_{12} and the inhibitor to the assay mixture. K_i of aristeromycylcobalamin calculated by this method is 16 ± 2 nM.

In the methylmalonyl-CoA mutase reaction the value of K_i for aristeromycylcobalamin is ≈ 14 times higher than the value of K_M for AdoCbl and this reveals a weaker binding.

The analogue 2′,3′-bis-*epi*-aristeromycylcobalamin (**13**) was neither a coenzyme nor an inhibitor for any of the four enzymes tested. This indicated that the correct steric position of the ribose hydroxyl groups is required for the coenzyme to bind.

EPR spectroscopy: Substrates, products and their analogues convert the EPR silent adenosylcobalamin in adenosylcobalamin-dependent dehydratases and mutases into EPR-active species at a catalytically competent rate.[8–10, 16, 31, 32] Simulation of the EPR spectra has provided evidence for the presence of cob(II)alamin that is coupled to a carbon-centred radical 7 Å away in the mutases[14] and >10 Å away in the dehydratases.[33, 34] These predictions were corroborated by the X-ray crystallographic studies of glutamate mutase and dioldehydratase.

On incubation for less than 30 s of glyceroldehydratase from *Citrobacter freundii* (overexpressed in *E. coli*) with racemic 1,2-propanediol (100 mM) and the coenzymes AdoCbl, aristeromycylcobalamin (**12**) and [6′-^{13}C]aristeromycylcobalamin ([6′-^{13}C](**12**)), the "doublet" EPR feature that is characteristic of the organic radical partner of the cob(II)alamin-radical pair was observed (Figure 2). The features, two

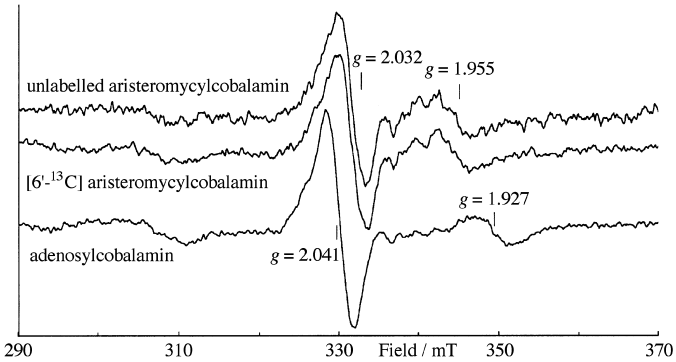

Figure 2. EPR spectroscopy of glyceroldehydratase (*C. freundii*) incubated with glycerol and coenzyme B_{12}, [^{13}C]-labelled and unlabelled aristeromycylcobalamin. $T = 77$ K; microwave power, 1.2 mW; modulation amplitude, 1.0 mT; receiver gain, 5×10^4; frequency, 9.431 GHz.

sharp derivative-shaped lines with a $P_{1/2}$ of ≈ 10 mW at 77 K, compare well with those of the dehydratase type of coupled spectra, in particular with those of the glyceroldehydratase from *Klebsiella pneumoniae* ATCC 25955 (formerly *Aerobacter aerogenes*, PZH 572)[31]. In addition, broad signals with g values between 2.3 and 2 were seen, these signals are assigned to the low spin Co^{II} of the B_{12} present in the enzyme-substrate complex. The exact positions of the derivative-shaped features of the doublet system are $g = 2.041 \pm 0.001$ and $g = 1.927 \pm 0.003$ for AdoCbl and at $g = 2.032 \pm 0.001$ and $g = 1.955 \pm 0.003$ for aristeromycylcobalamin (**12**) and [6′-^{13}C]aristeromycylcobalamin ([6′-^{13}C](**12**)). The linewidths for aristeromycylcobalamin (**12**) and [6′-^{13}C]aristeromycylcobalamin ([6′-^{13}C](**12**)) are identical within experimental error but are significantly larger than those obtained with AdoCbl. Also the ratio of intensity between the low and high field feature is lower (2.6:1) than in the spectrum with AdoCbl (4.1:1). This ratio is sensitive to both angular constants and the distance between Co^{II} and the radical as reflected by the coupling constant.[33] One can conclude that the geometry of the complex with aristeromycylcobalamin is similar but has subtle differences with respect to that with adenosylcobalamin. It seems that this distance becomes shorter when glyceroldehydratase is activated by aristeromycylcobalamin. This again suggests that the small geometrical difference between the furanose and cyclopentane ring has a large influence on the transition state of the B_{12}-dependent enzymic reactions. No other signals from a (coupled) intermediate adenosyl radical have been observed.

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Conclusion

Enantiomerically pure aristeromycin (**8**) and its [6′-^{13}C]isotopomer have been synthesized in seven steps from the commercially-available (1*S*,4*R*)-*cis*-acetoxycyclopent-2-ene-1-ol (**1**). The stereoselectivity of key substitution steps was increased with Pd^0 catalysts and polymer-bound triphenylphosphine to facilitate the purification procedures. To the best of our knowledge, this is the shortest synthesis of aristeromycin that introduces an isotopic label at the 6′-position. Aristeromycin (**8**) and its 2′,3′-bis-epimer (**9**) as well as their [6′-^{13}C]isotopomers have been coupled to cobalamin via their 6′-chloroderivatives **10** and **11**. Both coenzyme B_{12} analogues were assayed for their activity as cofactors of four coenzyme B_{12}-dependent enzymes. Aristeromycylcobalamin (**12**) had 44% and 38% of the activity of AdoCbl with dioldehydratase and glyceroldehydratase, respectively, although its K_M values were similar to those of AdoCbl.

In the cases of the enzymes methylmalonyl-CoA mutase and ethanolamine ammonia-lyase, aristeromycylcobalamin (**12**) did not serve as a coenzyme, but was a weak inhibitor for the former and a very strong inhibitor for the latter. EPR measurements with glyceroldehydratase and racemic propane-1,2-diol as substrate revealed small but significant changes of the doublet signal in comparison with those obtained using AdoCbl. However no difference in the EPR spectra was observed when unlabelled (**12**) and [6′-^{13}C]aristeromycylcobalamin ([6′-^{13}C](**12**)) was used as coenzyme and this indicates that the putative intermediate 6′-aristeromycyl radical has a too short lifetime to be detectable or that the 6′-^{13}C is too far away to broaden the signal.

Experimental Section

Materials and methods: Yeast alcohol dehydrogenase (ADH), L-malate/NAD oxidoreductase (MDH) coenzyme A and β-NADH Li_3 (NADH) were obtained from Roche Diagnostics Mannheim. Coenzyme B_{12}, hydroxycobalamin (vitamin B_{12a}), racemic 1,2 propanediol, succinic anhydride and ethanolamine hydrochloride were products of Fluka Chemie AG. Compounds (1*S*, 4*R*)-*cis*-acetoxycyclopent-2-ene-1-ol (diastereomeric purity $\geq$98%) and [^{13}C]nitromethane ([^{13}C]enrichment $\geq$99%) were purchased from Sigma-Aldrich. All other chemicals used are commercially available and were of highest purity. All solvents were freshly distilled and dried prior to use. For kinetic measurements coenzyme B_{12} and the aristeromycylcobalamin analogues were dissolved in bidistilled water and kept at 0–4 °C in the darkness. Under these conditions the solutions were stable for months (>98% pure from analytical HPLC).

Bacterial strains, cell cultivation and enzyme isolation

Ethanolamine ammonia-lyase: *E. coli* cells BL21 (DE3) were used as host. The ethanolamine ammonia-lyase gene from *Salmonella typhimurium* was inserted into a gene coding for a pT7-7 vector. The cells were cultivated at 30 °C for 24 h on Luria–Bertani agar plates which contained ampicillin and kanamycin. One colony was transferred into 50 mL Luria–Bertani media and incubated at 30 °C for 16 h. From this culture, a 10 mL portion was added to 1 L Luria–Bertani media and shaken at 37 °C for 2–4 h until the A_{600} reached 1.0. The expression of the enzyme was induced by the addition of IPTG (1 mM) and the cells were incubated at 37 °C for another 4 h. The cells were harvested by centrifugation at 5000 × *g* for 10 min and stored at −72 °C until use.

The enzyme was isolated from a cellfree extract as described by Röther[35] and further purified by ammonium sulphate precipitation (with no loss of enzyme activity), gel filtration chromatography and anion-exchange chromatography. The final enzyme concentration of 0.18 mg mL^{-1} (20 mL in 50% glycerol) was calculated by the method of Warburg & Christian. The specific activity of the enzyme was 24 U mg^{-1}.

Glyceroldehydratase: Host cells of *E. coli* HMS174 cells with a pQE30 vector from Qiagen which contained the gene for the glyceroldehydratase from *Citrobacter freundii*. The enzyme had a His_6-Tag introduced at the N-terminus of the enzyme.[36] The cells were cultured and harvested following as described above for those containing the ethanolamine ammonia-lyase vector, but expression was induced with only 10 μM IPTG. The enzyme was isolated and purified from a cellfree extract by immobilized metal affinity chromatography according to standard protocols.[37] The enzyme solution was concentrated to 800 μL and the final protein concentration was calculated from ($1.55 - 0.76$, $A_{280\,nm} - A_{260\,nm}$) to be 21 mg mL^{-1}. The preparation had a specific activity of 129 U mg^{-1} with racemic 1,2-propanediol as substrate.

Dioldehydratase: The dioldehydratase gene from *Salmonella typhimurium* was inserted into a pT7-7 vector and overexpressed in *E.coli* BL21. The cells were cultured and harvested as described above for ethanolamine ammonia-lyase. Dioldehydratase was isolated and purified from to the protocol described in the literature.[38] After disruption of the cells by sonication, the sediment was dissolved by the addition of the sodium salt of cholic acid and the resulting supernatant was applied to a HPLC gel filtration column (Superdex 200). The eluted enzyme was concentrated to 1 mL and had a concentration of 11 mg mL^{-1} The specific activity was 95 U mg^{-1} using racemic 1,2-propanediol as substrate.

Methylmalonyl-CoA mutase: Methylmalonyl-CoA mutase (EC 5.4.99.2), methylmalonyl-CoA epimerase (EC 5.1.99.1) and methylmalonyl-CoA transcarboxylase (EC 2.1.3.1) were isolated from *P. shermanii* according to previously published methods.[39, 40] Succinyl-CoA was freshly prepared.[29]

Kinetic measurements: Kinetic constants (K_M, k_{cat}) for adenosylcobalamin and aristeromycylcobalamin (**12**) were determined by means of standard linearization methods. 1–10 μL of 0.001–1 mM solutions of the cofactor were used to obtain 7–10 data points. Linearization was by the method of Lineweaver–Burk, Hanes and Eadie–Hofstee. From these three methods the average values of kinetic constants were calculated.

The inhibition constants (K_i) for the reaction of aristeromycylcobalamin with ethanolamine ammonia-lyase and methylmalonyl-CoA mutase were determined by the "parallel" method.[29] In this assay adenosylcobalamin and aristeromycylcobalamin were added simultaneously to the mixture. At three constant concentrations of adenosylcobalamin (0.5, 1 and 2.5 μM), the inhibitor concentration was varied (5–7 data points). Linear regression was performed by a Dixon plot, in which the aristeromycylcobalamin concentration is rated to 1/*v*. This results in three lines whose point of intersection when projected to the [aristeromycylcobalamin]-axis corresponds directly to K_i.

All following enzymatic assays were performed in the darkness and with degassed buffers.

Ethanolamine ammonia-lyase: A coupled assay with yeast alcohol dehydrogenase (ADH) was employed from the method of Blackwell and Turner[41] with some modifications. In a plastic cuvette, ethanolamine hydrochloride (10 mM) in 50 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.5, was mixed with NADH (30 mM, 10 μL), ADH (5 U, 5 μL) and the appropriate amount of ethanolamine ammonia-lyase (usually 2–3 μL of the prepared solution). The mixture was incubated at 37 °C for 5 min and the enzymic reaction was started by addition of coenzyme B_{12}. The decrease of absorbance at 340 nm was recorded for 5 min at 37 °C. The rate of ethanolamine transformation to acetaldehyde was calculated from the change of absorption at 340 nm, which is due to the consumption of NADH in the coupled enzyme assay. Inhibition kinetics were performed with the same assay using the "parallel" method.[29]

Glyceroldehydratase and dioldehydratase: The ADH/β-NADH coupled assay used was based on the method of Bachovchin et al.[42] with some minor modifications. In a plastic cuvette, 0.1% racemic 1,2-propanediol in 20 mM K_2HPO_4/KH_2PO_4 buffer, pH 8.0, was mixed with NADH (30 mM, 10 μL), ADH (5 U, 5 μL) and the appropriate amount of glycerol- or dioldehydratase (usually 2–3 μL of a 1:100 dilution of the concentrated protein solution). The mixture was incubated at 37 °C for 5 min and the enzymic reaction was started by addition of coenzyme B_{12}. The decrease of absorbance at 340 nm was recorded for 5 min at 37 °C.

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Methylmalonyl-CoA mutase: The enzyme assay was based on the method of Zagalak et al.[40] with some minor changes. In a microcuvette, sodium pyruvate (0.1 M, 70 μL), NADH (30 mM, 10 μL), MDH (30 U mL^{-1}, 10 μL) and succinyl-CoA (20 μL, 20 mmol) in Tris-HCl-buffer, pH 7.5 (0.1 M, 765 μL,) were mixed with epimerase (3 U mL^{-1}, 20 μL), transcarboxylase (5 U mL^{-1}, 5 μL,) and mutase (4.5 U mL^{-1}, 5 μL). The mixture was incubated at 30 °C for 5 min and the enzymic reaction was started by addition of coenzyme B_{12}. The decrease of absorbance at 340 nm was recorded for 5 min at 30 °C. Inhibition kinetics were determined by the "parallel" method.[29]

EPR spectroscopy: The kinetically-competent EPR-detectable "doublet" intermediate[31, 32] was generated by the incubation of *Citrobacter freundii* glyceroldehydratase with racemic 1,2-propanediol. Trapping of this intermediate was performed by manually freezing reaction mixtures in ethanol which was cooled close to its melting point with liquid nitrogen. This required skill but produced similar quantities of the EPR-active species[32] and avoided the problems of trapped, solid oxygen, complicated storage and loss of sensitivity due to interstitials in the snow produced by rapid-freeze.

EPR tubes that were preloaded with buffer, glyceroldehydratase and adenosylcobalamin, [6′-^{13}C]-labelled or unlabelled aristeromycylcobalamin were cooled on ice. Generation of the doublet species was initiated by the addition of an ice-cold solution of racemic 1,2-propanediol. Transfer and mixing was done with a Gilson pipette with a 15 cm piece of Tefzel tubing (∅ 0.75 mm) linked to a yellow tip with silicon tubing (∅ 1 mm), which were all precooled in ice-cold buffer. The aerobic reaction mixtures had a final volume of 250 μL, and had final concentrations of 0.4 M racemic 1,2-propanediol, 5 mg mL^{-1} glyceroldehydratase, 4 mM adenosylcobalamin, and [6′-^{13}C]-labelled or unlabelled aristeromycylcobalamin in 20 mM K_2HPO_4/KH_2PO_4 buffer, pH 8.0. To avoid depletion of 1,2-propanediol and the generation of detrimental amounts of propionaldehyde, the mixing was performed as rapidly as possible: fastest mixing, transfer into the liquid nitrogen-cooled viscous ethanol and freezing took between 10–15 s in total. Control experiments with freezing after 25–45 s showed > 3 and > 10 fold reduction in the intensity of the doublet signal. The experiment was carried out under dim red light to avoid formation of hydroxycobalamin by photolysis.

Samples were stored in the dark in liquid nitrogen. No significant loss of signal intensity occurred upon storage for several months. The EPR measurements were performed with a Bruker EMX-6/1 X-band EPR Spectrometer with a built-in ER-041-1161 microwave frequency counter, EMX-1101 magnet and power supply composed of an ER-070 6 inch magnet with 60 mm air gap, an EMX-080 1 kW magnet power supply, an EMX-032 T Hall Field probe with electronics to interface with the magnet and an ER-4102 standard Universal TE 102 rectangular cavity with a typical resonance of ≈9.431 GHz. Initial experiments were performed using a liquid nitrogen finger Dewar. The spectra presented in this paper were recorded at 77 K using the ESR-900 Oxford Instruments variable temperature Helium flow cryostat. Data acquisition was with the software supplied by Bruker (WINEPR Acquisition program, May 1, 1997, version 2.3.1.), data manipulation (determination of *g* values, subtraction, baseline correction and conversion to ASCII files for use with Microsoft EXCEL) was done with the WINEPR program version 2.11. EPR tubes were 4.7 ± 0.2 mm outer diameter (0.45 ± 0.05 mm wall thickness) and 13 cm length Ilmasil-PN high purity quartz tubes obtained from Quarzschmelze Ilmenau GmbH (Langewiesen, Germany).

Synthetic part

General methods: Melting points were recorded on a Büchi 535 melting point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were both recorded at 500 MHz and 125 MHz, respectively, with a Bruker DRX 500. Spin multiplicities are indicated by the symbols s (singlet), d (doublet), dd (doublet of doublet), t (triplet), dt (doublet of triplet), m (multiplet) and dm (doublet of multiplet). IR spectra were recorded with a Bruker IFS 88 FT-IR spectrometer using the DRIFT-method (Diffuse Reflectance Infrared Fourier Transform). EI-MS (electron impact) was performed with a Finnigan-MAT-90 high-resolution instrument at 70 eV. Reactions were monitored by TLC (thin layer chromatography) using silica gel precoated aluminium plates G_{254} from Macherey & Nagel. All substances could be visualized by irradiation with a UV lamp. Column flash chromatography was performed on silica gel 60 (40–63 μm grade) by Merck, and elution was done with the indicated solvent systems. HPLC-separations were done with a Merck Hitachi L-6210 pump, an L-4000 UV detector, a D-2500 chromato-integrator, an analytical Nucleosil-10-C_{18} column (250 × 4 mm) and a preparative Nucleosil-7-C_{18} column (250 mm × 1″) from Macherey & Nagel.

Synthesis of [6′-^{13}C]aristeromycin (8)

(1′*R*,4′*S*)-4′-Benzoyloxy-1′-(6-chloro-9*H*-purin-9-yl)cyclopent-2′-ene (2): Polymer-bound triphenylphosphine (1.17 g, 3 mmol/g) and $Pd_2(dba)_3 \cdot CHCl_3$ (364 mg, 0.35 mmol) was added to a deoxygenated mixture of (1*S*,4*R*)-*cis*-acetoxycyclopent-2-ene-1-ol (**1**) (1 g, 7.03 mmol) and chloropurine (2.17 g, 14.06 mmol) in THF (30 mL). The mixture was stirred for 10 min until the colour changed from deep red to yellow. Triethylamine (5 mL) was added and the mixture was heated under reflux at 57 °C for 15 h. The solution was cooled to room temperature and the polymer-bound triphenylphosphine was filtered off. Benzoylchloride (2.5 mL, 21.5 mmol) was added and the mixture was stirred at room temperature for 17 h. It was then concentrated and diluted with diethyl ether. The organic layer was washed with water, dried (with $MgSO_4$) and concentrated. The residue was purified by flash chromatography (ethyl acetate/hexane 1:1) to give **2** as a white foam (1.44 g, 60 %). $R_f = 0.26$; ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C): $\delta = 8.77$ (s, 1H, (2)-CH), 8.27 (s, 1H, (8)-CH), 8.00–7.98 (m, 2H, phenyl-H), 7.57–7.44 (m, 3H, phenyl-H), 6.55–6.53 (m, 1H, (3′)-CH), 6.28 (dd, 1H, $J_1 = 6$ Hz, $J_2 = 2$ Hz, (2′)-CH), 6.03 (dd, 1H, $J_1 = 5$ Hz, $J_2 = 2$ Hz, (4′)-CH), 5.86–5.84 (m, 1H, (1′)-CH), 3.14 (dt, 1H, $J_1 = 16$ Hz, $J_2 = 8$ Hz, (5′)-CH_2), 2.13 (dt, $J_1 = 15$ Hz, $J_2 = 3$ Hz, (5′)-CH_2); ^{13}C NMR (125 MHz, $CDCl_3$, 25 °C): $\delta = 165.90$ (phenylester-C), 152.06 ((2)-CH), 151.34 ((6)-C), 151.14 ((4)-C), 143.41 ((5)-C), 136.68 ((8)-CH), 133.60 ((3′)-CH), 133.44 ((2′)-CH), 130.04 (phenyl-CH), 129.55 (2 × phenyl-CH), 129.43 (phenyl-CH), 128.60 (2 × phenyl-CH), 77.34 ((4′)-CH), 57.53 ((1′)-CH), 38.67 ((5′)-CH_2); MS (EI, 70 eV, 140 °C): *m*/*z* (%): 341 (6) [M^+], 219 (22), 218 (7), 194 (9), 192 (38), 105 (100), 77 (22), 65 (9); HRMS (EI): *m*/*z*: calcd for $C_{17}H_{13}Cl_1N_4O_2$: 340.7636 [M^+]; found: 340.7625; IR ($CDCl_3$): $\tilde{\nu} = 3411$, 3068, 2987, 1717, 1592, 1565, 1487, 1454, 1436, 1402, 1369, 1334, 1318, 1296, 1271, 1215, 1170, 1148, 1132, 1100, 1070, 1045, 1026, 1001, 952, 926, 883, 863, 855, 836, 800, 790, 749, 720, 686, 670, 651, 637 cm^{-1}.

Phenylsulphonyl[^{13}C]nitromethane (3): [^{13}C]Nitromethane (250 mg, 4.1 mmol) in DMF (5 mL) was cooled to 0 °C. Then 1,8-diazabicyclo[5.4.0]undec-7-ene (620 μL, 4.10 mmol) was added and the reaction mixture was stirred for 10 min. After addition of sodium phenylsulphinate (560 mg, 3.42 mmol) and iodine (786 mg, 3.11 mmol), the solution was allowed to warm to room temperature and stirred for 1 h. A saturated solution of sodium sulphite was added to the solution until the colour of the mixture turned to bright yellow to remove the iodine. The pH was adjusted to 1 with concentrated HCl. Then the aqueous layer was extracted three times with diethyl ether and the organic layer was dried ($MgSO_4$), concentrated and purified by flash chromatography (ethyl acetate/hexane 1:3) to give white crystals (330 mg, 40 %). M.p. 78 °C; $R_f = 0.18$; ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C): $\delta = 7.93–7.87$ (m, 2H, phenyl-H), 7.74–7.70 (m, 1H, phenyl-H), 7.62–7.55 (m, 2H, phenyl-H), 5.53 (d, 2H, $J = 156$ Hz, $PhSO_2$-$^{13}CH_2$-NO_2); ^{13}C NMR (125 MHz, $CDCl_3$, 25 °C): $\delta = 135.71$ (d, $J =$ 9 Hz, quart. C), 135.54 (phenyl-CH), 129.70 (2 × phenyl-CH), 129.29 (2 × phenyl-CH), 90.29 (^{13}C label); MS (EI, 70 eV, 45 °C): *m*/*z* (%): 202 (20) [M^+], 158 (8), 141 (56), 94 (6), 78 (5), 77 (100), 51 (16); HRMS (EI): *m*/*z*: calcd for $^{13}C_1C_6H_7N_1O_4S_1$: 202.2008 [M^+]; found: 202.2012; IR ($CDCl_3$): $\tilde{\nu} = 3064$, 3019, 2951, 2732, 2581, 2472, 2240, 1972, 1910, 1821, 1551, 1449, 1350, 1325, 1210, 1154, 1085, 1024, 998, 933, 918, 742, 698, 682 cm^{-1}.

(1′*R*,4′*S*)-1′-(6-Chloro-9*H*-purin-9-yl)-4′-[nitro(phenylsulphonyl)-[^{13}C]-methyl]cyclopent-2′-ene (4): Polymer-bound triphenylphosphine (227 mg, 3 mmol/g) and $Pd_2(dba)_3 \cdot CHCl_3$ (70 mg, 0.068 mmol) were added to a deoxygenated solution of the benzoate **2** (464 mg, 1.36 mmol) and phenylsulphonyl[^{13}C]nitromethane (**3**) (330 mg, 1.63 mmol) in THF (20 mL). The mixture was stirred for 10 min until the colour changed from deep-red to yellow. Then triethylamine (1.5 mL) was added and the mixture was stirred at room temperature for 15 h. The polymer-bound triphenylphosphine was filtered off and the solution was concentrated. The residue was diluted in saturated aqueous ammonium chloride. The aqueous layer was extracted three times with chloroform, then the organic layer was dried ($MgSO_4$) and concentrated. The residue was purified by flash chromatography (ethyl acetate/hexane 3:1) to give **4** as a white foam (503 mg, 88 %). $R_f = 0.34$; ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C): $\delta = 8.68$ (s, 2H, (2)-CH), 8.12 (s, 2H, (8)-CH), 7.93–7.60 (m, 10H, phenyl-H), 6.68–6.26 (dm, 2H, $J = 145$ Hz,

(6′)-^{13}CH), 6.54–6.52 (m, 1H, (2′)-CH), 6.03–5.99 (m, 2H, (3′)-CH, (2′)-CH), 5.90–5.88 (m, 1H, (3′)-CH), 5.80–5.75 (m, 1H, (1′)-CH), 5.65–5.62 (m, 1H, (1′)-CH), 4.01–3.82 (m, 2H, (4′)-CH), 3.22–3.13 (m, 1H, (5′)-CH_2), 3.05–2.95 (m, 1H, (5′)-CH_2), 2.47–2.40 (m, 1H, (5′)-CH_2), 2.15–2.09 (m, 1H, (5′)-CH_2); ^{13}C NMR (125 MHz, $CDCl_3$, 25 °C): δ = 151.72 (2 × (6)-C), 151.12 (2 × (4)-C), 143.83 (2 × (2)-C), 135.82 (2 × (8)-C), 134.37 ((2′)-CH), 134.32 ((2′)-CH), 134.17 (2 × (5)-C), 132.34 ((3′)-CH), 132.21 ((3′)-CH), 132.09 (2 × phenyl-C), 129.78 (2 × phenyl-CH), 129.76 (phenyl-CH, 4 x), 129.74 (4 × phenyl-CH), 102.80 (^{13}C label), 61.13 ((1′)-CH), 59.95 ((1′)-CH), 44.17 (d, J = 34 Hz, (4′)-CH), 43.84 (d, J = 34 Hz, (4′)-CH), 33.93 ((5′)-CH_2), 33.64 ((5′)-CH_2); MS: (FAB, DMSO/glycerol): m/z (%): 421 (5) [M^+], 405 (3), 386 (10), 298 (15), 185 (100), 93 (90); HRMS (EI): m/z: calcd for $^{13}C_1C_{16}H_{14}Cl_1N_5O_4S_1$: 420.8431 [$M^+$]; found: 420.8439; IR ($CDCl_3$): $\tilde{\nu}$ = 3412, 3271, 3062, 3032, 2926, 1969, 1902, 1819, 1618, 1579, 1558, 1495, 1478, 1448, 1438, 1406, 1335, 1300, 1247, 1221, 1179, 1157, 1119, 1082, 1028, 998, 971, 911, 874, 842, 796, 754, 721, 696, 686, 648 cm^{-1}.

(1′*R*,4′*S*)-1′-(6-Chloro-9*H*-purin-9-yl)-4′-methoxy[^{13}C]carbonylcyclopent-2′-ene (5): Nitrosulphonate **4** (400 mg, 0.95 mmol) was diluted in deoxygenated methanol (15 mL). Anhydrous sodium carbonate (1 g, 9.5 mmol) was added and the mixture was stirred vigorously for 15 min at room temperature. A solution of tetrabutylammonium oxide (4.64 g, 2.85 mmol) in dichloromethane (10 mL) was added slowly to the reaction mixture and then stirred at room temperature for another 15 h. The solution was concentrated and the residue was taken up in saturated aqueous ammonium chloride. The aqueous layer was extracted five times with diethyl ether and the combined organic layers were dried ($MgSO_4$) and concentrated. The residue was purified by flash chromatography (ethyl acetate/hexane 9:1) to give **5** as white crystals (191 mg, 72%). R_f = 0.41; ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C): δ = 8.72 (s, 1H, (2)-CH), 8.24 (s, 1H, (8)-CH), 6.32–6.30 (m, 1H, (2′)-CH), 6.05–6.01 (m, 1H, (3′)-CH), 5.91–5.85 (m, 1H, (1′)-CH), 3.71 (s, 3H, O-CH_3), 3.80–3.69 (m, 1H, (4′)-CH), 2.98–2.85 (m, 1H, (5′)-CH_2), 2.35–2.26 (m, 1H, (5′)-CH_2). ^{13}C NMR (125 MHz, $CDCl_3$, 25 °C): δ = 173.08 (^{13}C label), 151.79 ((6)-C), 151.43 ((4)-C), 150.93 ((2)-CH), 143.92 ((8)-CH), 136.06 ((2′)-CH), 131.75 ((3′)-CH), 130.44 ((5)-C), 59.33 ((1′)-CH), 52.49 (O-CH_3), 49.43 (d, J = 58 Hz, (4′)-CH), 34.24 ((5′)-CH_2); MS (EI, 70 eV, 105 °C): m/z (%): 280 (12) [M^+], 279 (44), 247 (10), 219 (33), 194 (10), 192 (37), 157 (28), 155 (100), 125 (20), 124 (29), 93 (28), 66 (45), 65 (48); HRMS (EI): m/z: calcd for $^{13}C_1C_{11}H_{11}Cl_1N_4O_2$: 279.6942 [$M^+$]; found: 279.6951; IR ($CDCl_3$): $\tilde{\nu}$ = 3433, 3113, 3057, 2992, 2950, 1852, 1727, 1591, 1559, 1491, 1436, 1424, 1402, 1337, 1316, 1253, 1200, 1149, 1134, 1119, 1066, 1003, 958, 928, 911, 858, 791, 769, 741, 686, 652, 637, 627 cm^{-1}.

(1′*R*,4′*S*)-1′-(6-Chloro-9*H*-purin-9-yl)-4′-hydroxy[^{13}C]methylcyclopent-2′-ene (6): The methylester **5** (150 mg, 0.54 mmol) in deoxygenated THF (15 mL) was cooled in dry ice/acetone bath to −78 °C. Superhydride ($LiHB(Et)_3$) (1.62 mL of a 1M solution) was added dropwise and very slowly over 30 min as the reaction mixture was stirred vigorously. After stirring for 3 h at −78 °C, the reaction was quenched with ethyl acetate (10 mL) and saturated aqueous ammonium chloride (15 mL) and warmed to room temperature. The aqueous phase was extracted three times with ethyl acetate. The combined organic layers were washed with water, dried ($MgSO_4$) and concentrated. The residue was purified by flash chromatography (ethyl acetate/methanol 24:1) to give **6** as a yellow gum (97 mg, 72%). R_f = 0.38; ^{1}H NMR (500 MHz, MeOD, 25 °C): δ = 8.70 (s, 1H, (2)-CH), 8.44 (s, 1H, (8)-CH), 6.23–6.17 (m, 1H, (1′)-CH), 5.89–5.82 (m, 1H, (2′)-CH), 5.80–5.71 (m, 1H, (3′)-CH), 3.65–3.87 (dm, 2H, J = 122 Hz, (6′)-$^{13}CH_2$), 3.18–3.02 (m, 1H, (5′)-CH_2), 2.90–2.78 (m, 1H, (4′)-CH), 1.98–1.85 (m, 1H, (5′)-CH_2). ^{13}C NMR (125 MHz, MeOD, 25 °C): δ = 151.62 ((4)-C), 151.59 ((6)-C), 150.66 ((2)-CH), 144.85 ((8)-CH), 139.91 ((2′-CH), 131.61 ((3′-CH), 129.09 ((5)-C), 64.18 ($^{13}CH_2$ label), 60.66 ((1′)-CH), 47.59 ((4′)-CH), 33.86 ((5′)-CH_2); MS (EI, 70 eV, 220 °C): m/z (%): 252 (10) [M^+], 151 (23), 98 (100), 71 (19), 70 (15), 66 (34), 45 (12); HRMS (EI): m/z: calcd for $^{13}C_1C_{10}H_{11}Cl_1N_4O_1$: 251.6841 [$M^+$]; found: 251.6838; IR (MeOD): $\tilde{\nu}$ = 3265, 3059, 2960, 1900, 1692, 1607, 1591, 1531, 1495, 1476, 1450, 1404, 1346, 1328, 1300, 1226, 1196, 1160, 1119, 1064, 1028, 998, 972, 911, 858, 794, 721, 696, 632 cm^{-1}.

(1′*R*,4′*S*)-1′-(6-Amino-9*H*-purin-9-yl)-4′-hydroxy[^{13}C]methylcyclopent-2′-ene (7): The alcohol **6** (80 mg, 0.32 mmol) was dissolved in a mixture of THF (3 mL) and concentrated aqueous ammonia (6 mL). The flask was sealed and stirred for 72 h. The mixture was concentrated and directly purified by flash chromatography (ethyl acetate/methanol 3:1) to give **7** as a white solid (64 mg, 87%). R_f = 0.38; ^{1}H NMR (500 MHz, [D$_6$]DMSO, 25 °C): δ = 8.21 (s, 1H, (2)-CH), 8.12 (s, 1H, (8)-CH), 7.29 (s, 2H, -NH_2), 6.21–6.19 (m, 1H, (1′)-CH), 5.98–5.94 (m, 1H, (2′)-CH), 5.71–5.65 (m, 1H, (3′)-CH), 4.95 (s, 1H, -OH), 3.55–3.82 (dm, 2H, J = 134 Hz, (6′)-$^{13}CH_2$), 3.03–2.96 (m, 1H, (5′)-CH_2), 2.79–2.70 (m, 1H, (4′)-CH), 1.81–1.75 (m, 1H, (5′)-CH_2); ^{13}C NMR (125 MHz, [D$_6$]DMSO, 25 °C): δ = 150.87 ((4)-C), 150.12 ((6)-C), 148.24 ((2)-CH), 142.98 ((8)-CH), 139.45 ((2′-CH), 131.35 ((3′-CH), 130.09 ((5)-C), 63.95 ($^{13}CH_2$-label), 60.95 ((1′)-CH), 47.55 ((4′)-CH), 34.76 ((5′)-CH_2; MS (EI, 70 eV, 210 °C): m/z (%): 233 (11) [M^++H], 140 (9), 135 (51), 136 (100), 99 (22), 43 (52); HRMS (EI): m/z: calcd for $^{13}C_1C_{10}H_{13}N_5O_1$: 232.2540 [$M^+$]; found: 232.2534; IR (DMSO): $\tilde{\nu}$ = 3320, 2905, 1650, 1601, 1478, 1414, 1298, 1252, 1221, 1109, 915, 796, 678 cm^{-1}.

(1′*R*,2′*S*,3′*R*,4′*S*)-1′-(6-Amino-9*H*-purin-9-yl)-4′-hydroxy[^{13}C]methylcyclopentane-2′,3′-diol; 6′-[^{13}C]aristeromycin (8) and (1′*R*,2′*R*,3′*S*,4′*S*)-1′-(6-amino-9*H*-purin-9-yl)-4′-hydroxy[^{13}C]methylcyclopentane-2′,3′-diol; 2′,3′-bis-*epi*-6′-[^{13}C]aristeromycin (9): Methylmorpholine-*N*-oxide (51 mg, 0.44 mmol) was added to a solution of the unsaturated alcohol **7** (50 mg, 0.22 mmol) in water (2 mL) and THF (4 mL). After the mixture was cooled to 0 °C, a catalytic amount of solid osmium tetroxide (10 mg, 0.04 mmol) was added and the solution was stirred for 24 h at 0 °C. The reaction mixture was quenched by the dropwise addition of saturated aqueous sodium sulphite. After warming to room temperature and stirring for an additional 30 min, the brown solution was directly applied to a silica gel column and purified by flash chromatography (ethyl acetate/methanol 3:1) to give 72 mg of a mixture of **8** and **9** as a white solid (R_f = 0.30). The crude product was still contaminated with NMO and it was nearly impossible to separate the two diastereomers by simple flash chromatography. So the two products were further purified by preparative HPLC (λ 280 nm; ν = 4 mL min^{-1}; eluents: H_2O (A) and MeOH (B), using a linear gradient of 5–20% B within 120 min). [6′-^{13}C]Aristeromycin (**8**) eluted after 70 min as a white solid (28 mg, 0.11 mmol, 49%). M.p. 237 °C. 2′,3′-Bis-*epi*-6′-[^{13}C]aristeromycin (**9**) eluted after 82 min as a white solid (22 mg, 0.08 mmol, 38%). M.p. 226 °C.

6′-[^{13}C]Aristeromycin (**8**): ^{1}H NMR (500 MHz, [D$_6$]DMSO, 25 °C): δ = 8.17 (s, 1H, (2)-CH), 8.10 (s, 1H, (8)-CH), 7.13 (s, 2H, NH_2), 4.89 (d, 1H, J = 7 Hz, -OH), 4.71–4.64 (m, 3H, (1′)-CH, 2 × -OH), 4.34–4.30 (m, 1H, (2′)-CH), 3.82 (dd, 1H, J_1 = 8 Hz, J_2 = 4 Hz, (3′)-CH), 3.64–3.27 (dm, 2H, J = 140 Hz, (6′)-$^{13}CH_2$), 2.24–2.18 (m, 1H, (5′)-CH_2), 2.07–2.02 (m, 1H, (4′)-CH_2), 1.75–1.68 (m, 1H, (5′)-CH_2). ^{13}C NMR (125 MHz, [D$_6$]DMSO, 25 °C): δ = 155.90 ((6)-C), 152.00 ((2)-CH), 149.69 ((4)-C), 140.00 ((8)-CH), 119.30 ((5)-C), 74.53 ((2′)-CH), 71.64 ((3′)-CH), 63.00 ((6′)-$^{13}CH_2$), 59.26 ((1′)-CH), 45.31 (d, J = 38 Hz, (4′)-CH), 29.25 ((5′)-CH_2); MS (EI, 70 eV, 150 °C): m/z (%): 266 (4) [M^+], 162 (25), 137 (17), 136 (100), 135 (47), 108 (7), 43 (7); HRMS (EI): m/z: calcd for $^{13}C_1C_{10}H_{15}N_5O_3$: 266.2687 [$M^+$]; found: 266.2690; IR ([D$_6$]DMSO): $\tilde{\nu}$ = 3327, 2871, 1650, 1599, 1478, 1417, 1331, 1252, 1212, 1109, 913, 795, 723, 645 cm^{-1}.

2′,3′-Bis-*epi*-[^{13}C]aristeromycin (**9**):^{1}H NMR (500 MHz, [D$_6$]DMSO, 25 °C): δ = 8.15 (s, 1H, (2)-CH), 8.10 (s, 1H, (8)-CH), 7.12 (s, 2H, NH_2), 5.07 (d, 1H, J = 6 Hz, -OH), 4.99–4.89 (m, 2H, (1′)-CH, -OH), 4.50 (dd, 1H, J_1 = 8 Hz, J_2 = 4 Hz, (3′)-CH), 4.10–4.02 (m, 2H, (2′)-CH, -OH), 3.78–3.30 (dm, 2H, J = 180 Hz, (6′)-$^{13}CH_2$), 2.26–2.18 (m, 1H, (5′)-CH_2), 2.11–2.02 (m, 1H, (4′)-CH), 1.90–1.80 (m, 1H, (5′)-CH_2); ^{13}C NMR (125 MHz, [D$_6$]DMSO, 25 °C): δ = 156.30 ((6)-C), 152.35 ((2)-CH), 150.26 ((4)-C), 141.82 ((8)-CH), 118.75 ((5)-C), 74.24 ((2′)-CH), 72.51 ((3′)-CH), 60.99 ((6′)-$^{13}CH_2$), 53.59 ((1′)-CH), 40.44 (d, J = 35 Hz, (4′)-CH), 33.25 ((5′)-CH_2); MS (EI, 70 eV, 150 °C): m/z (%): 266 (25) [M^+], 234 (6), 190 (8), 178 (9), 162 (55), 148 (6), 136 (100), 135 (57), 108 (14), 58 (14), 43 (66); HRMS (EI): m/z: calcd for $^{13}C_1C_{10}H_{15}N_5O_3$: 266.2687 [$M^+$]; found: 266.2689; IR ([D$_6$]DMSO): $\tilde{\nu}$ = 3328, 3215, 1648, 1600, 1485, 1402, 1322, 1252, 1212, 1101, 901, 788, 723, 640 cm^{-1}.

(1′*R*,2′*S*,3′*R*,4′*S*)-1′-(6-Amino-9*H*-purin-9-yl)-4′-chloro[^{13}C]methylcyclopentane-2′,3′-diol, 6′-chloro-6′-deoxy-6′-[^{13}C]aristeromycin (10) and (1′*R*,2′*R*,3′*S*,4′*S*)-1′-(6-amino-9*H*-purin-9-yl)-4′-chloro[^{13}C]methylcyclopentane-2′,3′-diol, 2′,3′-bis-*epi*-6′-chloro-6′-deoxy-[6′-^{13}C]aristeromycin (11): [6′-^{13}C]Aristeromycin (**8**) (15 mg, 0.056 mmol) was dissolved in hexamethylphosphoramide (5 mL) and thionyl chloride (55 µL, 0.28 mmol) was added to the solution. The mixture was stirred at room temperature for 15 h and then the reaction mixture was quenched with water (15 mL). The product was purified by ion-exchange chromatography. The aqueous solution was directly applied to a column of Dowex 50 WX8

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(H^+ form) (10 mL). The column was washed with water (100 mL) and the product was eluted with 1 N aqueous ammonia (100 mL). After evaporating most of the solvent, the compound crystallized directly from the concentrated solution. The precipitation was separated and purified by repeated steps of centrifugation and washing with small amounts of water. 6′-Chloro-6′-deoxy-[6′-^{13}C]aristeromycin (**10**) was obtained as a white solid (13.4 mg, 0.047 mmol, 84 %).

The whole procedure was repeated with 2′,3′-bis-*epi*-[6′-^{13}C]aristeromycin (**9**) to obtain 2′,3′-bis-*epi*-6′-chloro-6′-deoxy-[6′-^{13}C]aristeromycin (**11**) in the same yield. The crude white products were used in the next reaction without further purification

[6′-^{13}C]Aristeromycylcobalamin (12) and its bis-*epi* isomer (13): Vitamin B_{12a} (hydroxycobalamin hydrochloride, 54 mg, 0.04 mmol) and a catalyic amount of cobalt(II) chloride (2 mg) were dissolved in deoxygenated water (4 mL). A solution of sodium borohydride (15 mg, 0.40 mmol) in deoxygenated water (1 mL) was added under argon and the resulting solution was stirred for 30 min. The deep-red colour of the mixture changed to brown and finally to greenish-grey. 6′-Chloro-6′-deoxy-6′-[^{13}C]aristeromycin (**10**; 11 mg, 0.04 mmol) in deoxygenated methanol (3 mL) was added through a syringe with exclusion of oxygen. The flask was kept dark and the mixture stirred vigorously at room temperature for 2 h. The solid precipitate (cobalt oxide) was separated from the mixture in the dark by centrifugation. The remaining deep-red solution was then directly purified by HPLC (λ 280 nm; $\nu = 4$ mL min^{-1}; eluents: CF_3COOH/H_2O (A) and MeOH (B), with a linear gradient of 20–80 % *B* within 60 min). [6′-^{13}C]aristeromycylcobalamin (**12**) eluted after 30 min as a yellow solution (the yellow colour was from the product in acidified solvent). Evaporation in the dark and drying in high vacuum yielded bright-red crystals (38 mg, 0.024 mmol, 60 %).

This procedure was repeated with 2′,3′-bis-*epi*-6′-chloro-6′-deoxy-[6′-^{13}C]aristeromycin (**11**) to obtain 2′,3′-bis-*epi*-[6′-^{13}C]aristeromycylcobalamin (**13**). This compound eluted after 35 min also as a yellow solution. Evaporation in the dark and drying in high vacuum yielded a bright-red solid ((34 mg, 0.022 mmol, 55 %).

[6′-^{13}C]Aristeromycylcobalamin (**12**): ^{1}H NMR (500 MHz, D_2O, 25 °C, only annotated data from Scheme 3 is presented: $\delta = 8.19$ (s, 1H, A2), 8.00 (s, 1H, A8), 7.19 (s, 1H, B7), 6.98 (s, 1H, B2), 6.26 (s, 1H, R1), 6.22 (s, 1H, B4), 5.95 (s, 1H, C10), 4.82 (m, 1H, A11), 4.78 (m, 1H, R3), 4.50 (m, 1H, A12), 4.35 (m, 1H, L2), 4.25 (m, 1H, R2), 4.12 (m, 1H, R4), 3.90 (m, 1H, R5), 3.75 (m, 1H, R5), 3.58 (m, 1H, L1), 3.45 (m, 1H, A13), 3.20 (m, 1H, L1), 2.22 (s, 3H, B10), 2.21 (s, 3H, B11), 2.12 (m, 1H, A16), 1.85 (m, 1H, A14), 1.82 (m, 1H, A15), 1.80 (m, 1H, A16), 1.21 (m, 3H, L3), 0.80 (m, 1H, A15); ^{13}C NMR (125 MHz, D_2O, 25 °C, only annotated data from Scheme 3 is presented: $\delta = 155.4$ (A6), 152.1 (A2), 148.8 (A4), 141.4 (B2), 140.5 (A8), 137.9 (B9), 133.6 (B6), 131.3 (B5), 130.2 (B8), 118.9 (A5), 118.4 (B4), 110.3 (B7), 94.7 (C10), 86.2 (R1), 76.2 (A11), 73.4 (A13), 72.8 (R3), 71.5 (L2), 60.0 (A12), 46.3 (A16), 43.9 (L1), 31.4 (^{13}C-A15), 30.2 (d, $J = 31$ Hz, A14), 18.7 (B10), 18.5 (B11), 18.1 (L3); characteristic data from the corrin moiety and its side chains is comparable with those of adenosylcobalamin as previously assigned;[43] MS (FAB, glycerol): *m*/*z* (%): 1578 (9) [M^+], 1561 (11), 1512 (10), 1478 (12), 1421 (15), 1330 (80) [M^+ – aristeromycyl-part], 1069 (14), 971 (22), 916 (17), 829 (15), 94 (100); calcd for $^{13}C_1C_{72}H_{102}Co_1N_{18}O_{16}P_1$: 1578.6097 [$M^+$].

2′,3′-Bis-*epi*-[6′-^{13}C]aristeromycylcobalamin (**13**): ^{1}H NMR (500 MHz, D_2O, 25 °C, only annotated data from Scheme 3 is presented): $\delta = 8.19$ (s, 1H, A2), 8.00 (s, 1H, A8), 7.19 (s, 1H, B7), 7.00 (s, 1H, B2), 6.29 (s, 1H, R1), 6.27 (s, 1H, B4), 6.02 (s, 1H, C10), 4.82 (m, 1H, A11), 4.75 (m, 1H, R3), 4.37 (m, 1H, A12), 4.35 (m, 1H, L2), 4.27 (m, 1H, R2), 4.15 (m, 1H, R4), 3.92 (m, 1H, R5), 3.78 (m, 1H, R5), 3.58 (m, 1H, L1), 3.29 (m, 1H, A13), 3.20 (m, 1H, L1), 2.25 (s, 3H, B10), 2.24 (s, 3H, B11), 2.15 (m, 1H, A16), 1.92 (m, 1H, A14), 1.87 (m, 1H, A15), 1.82 (m, 1H, A16), 1.25 (m, 3H, L3), 0.85 (m, 1H, A15); ^{13}C NMR (125 MHz, D_2O, 25 °C, only annotated data from Scheme 3 is presented): $\delta = 155.8$ (A6), 152.7 (A2), 149.2 (A4), 141.9 (B2), 141.1 (A8), 138.0 (B9), 133.8 (B6), 131.7 (B5), 130.3 (B8), 119.2 (A5), 118.5 (B4), 110.3 (B7), 94.6 (C10), 86.1 (R1), 78.6 (A11), 75.7 (A13), 72.5 (R3), 71.5 (L2), 63.2 (A12), 48.7 (A16), 43.7 (L1), 32.1 (^{13}C A15), 32.4 (d, $J = 36$ Hz, A14), 18.8 (B10), 18.5 (B11), 17.8 (L3). Remaining data from the corrin moiety and its side chains is comparable with those of adenosylcobalamin as previously assigned.[43] MS (FAB, glycerol): *m*/*z* (%): 1578 (6) [M^+], 1554 (10), 1463 (11), 1330 (49) [M^+ – aristeromycyl], 1183 (16), 1069 (12), 1053 (21), 971 (25), 843 (26), 207 (10), 94 (100); calcd for $^{13}C_1C_{72}H_{102}Co_1N_{18}O_{16}P_1$: 1578.6097 [$M^+$].

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