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A Peptide Dendrimer Model for Vitamin B_{12} Transport Proteins

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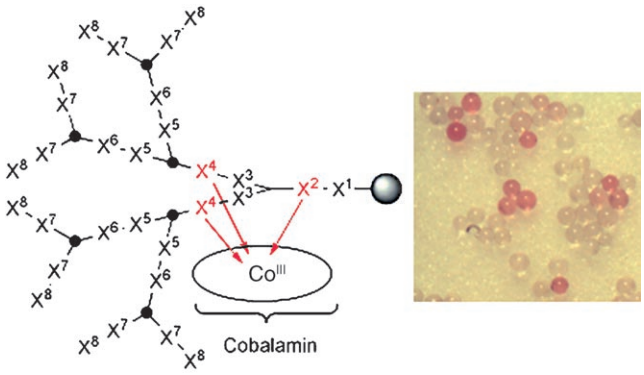
Natural proteins acquire their function by organized folding, which leads to the formation of active sites. Many proteins incorporate cofactors to complement their functionality. In the case of metal-containing cofactors such as porphyrins or cobalamins, the protein acts as a protecting shell around the cofactor; this leads to altered reactivity at the metal center. While porphyrin-containing proteins have been studied in a number of synthetic model systems,[1] no synthetic macromolecules have been reported that mimic the cobalt binding in B_{12}-dependent enzymes or transport proteins.[2] In a recent study, a monoclonal antibody against coenzyme B_{12} was reported that reconstitutes the "base-on" form of B_{12} coenzymes without direct coordination to the metal center.[3] A similar binding mode was reported in a selected aptamer ligand for cyanocobalamin.[4] Herein, we explored the molecular principles of cobalamin–peptidic ligand interaction with libraries of peptide dendrimers. Recognition and discrimination among a large number of amino acid sequences led to a peptide dendrimer model for a cobalamin-transport protein. In our peptidic ligands, cofactor binding is mediated by a coordinating cysteine or histidine residue at the dendrimer core and is assisted by secondary interactions in the dendritic shells.

Dendrimers are branched macromolecules that can adopt a globular or disk-shaped structure that is well suited to create synthetic models of proteins.[5] We recently reported peptide dendrimers that consist of natural amino acids arranged in a tree-like dendrimer topology by using a branching diamino acid at every second, third or fourth position in the peptide sequence.[6] These dendrimers display catalytic,[7] lectin binding[8] and drug-delivery activities.[9] While these functions resulted from multivalency effects of functional groups in the dendritic branches, the dendritic structure should also be well suited to engage in binding interactions at the dendritic core, which might form a tight complex in which a ligand could be secluded from solution by the surrounding dendritic branches, as was observed with porphyrins that had been functionalized with dendritic branches.[10] To test this hypothesis, we investigated binding to aquocobalamin, for which we had already discovered multivalent but only weakly coordinating dendritic ligands.[11] We prepared a split-and-mix combinatorial library A that displays nucleophilic residues such as histidine and cysteine at the core position X^2 and X^4 and charged, hydrophobic, aromatic and polar residues in the other variable positions (Figure 1).

Library A was tested for binding to aquocobalamin, and gave a few percent of darkly red-stained beads (Figure 1). Sequence determination revealed a consensus for cysteine residues at position X^2, together with an aspartate at X^1 or X^3, and



Library A (65'536 member)

X^8,X^6	X^7, X^5	X^4, X^2	X^3, X^1
Tyr	Thr	His	Ser
Ile	Pro	Arg	Asp
Trp	Phe	Cys	Leu
Glu	Gly	Val	Ala

● = *Dap*

Library B (9'216 member)

X^7, X^4	X^5, X^3
Gly, Arg	Thr, Tyr
Hyp, Ser	Pro, Val
Leu, b-Ala	Ala, Ile
Met, Amb	

● = *Dap/Lys*
X^1 = Asp
X^2 = Cys
$X^{6\,8}$ = Glu

Library C (7'560 member)

X^8	X^7	X^6	X^5	X^3
Ser	Thr	Amb	Met	Met
Tyr	Leu	Hyp	b-Ala	b-Ala
Ile	Val	Phe	Leu	Amb
Lys	Pro	Ile	Val	Hyp
Glu	Phe	Lys	Pro	Thr
	Gly	Glu	Tyr	Ser
			Gly	

● = *Dap*
X^1 = Ala
X^2 = Arg
X^4 = His

Figure 1. Combinatorial dendrimer libraries for cobalamin (Cbl) binding. One of the coordinating residues at X^2 or X^4 can coordinate to the metal center (red arrows). Insert top right: on-bead binding assay with aquocobalamin **1a**. Libraries were prepared on NovaSyn® TG resin (0.20–0.30 mmol g^{-1}; after attachment of 6-aminohexanoylglycine for library **A**), and the N termini were acetylated. Hyp = 4-hydroxyproline, b-Ala = β-alanine, Amb = 4-(aminomethyl)benzoic acid. The first branching unit between X^2 and X^3 is always Dap ((*S*)-2,3-diaminopropanoic acid).

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glutamate residues in the outer layers (X^8, X^6). The dendrimer sequence was optimized by testing a second focused library B, which incorporates further variations in amino acids and branching points, while keeping Cys-Asp at X^2X^1 and the glutamates at X^6X^8 constant (Figure 1, Figure 2). Dendrimers with a histidine residue that coordinates the cobalt were obtained from library C; these display a pair of histidines in position X^4 and Arg-Ala at X^2X^1 (Figures 1 and 2). The absence of His-containing dendrimers as hits from library A is probably due to the slower kinetics and lower binding affinity of imidazole derivatives to Co^{III} compared to thiols.[12] Screening of library A, B or C with cyanocobalamin **1b** or methylcobalamin **1c** (Figure 3A) gave no hits; this suggests that "base-off" coordination to the lower face by loop-displacement was not accessible from these libraries. Five cysteine-containing B_{12}-binding dendrimer hits, **A1**, **B1**–**B4**, one histidine-containing B_{12}-binding hit, **C1**, and one nonbinding sequence with histidine, **C2** were resynthesized, cleaved from the support, and studied in solution (Table 1). The peptide dendrimers that were resynthesized are representative of the screening results. In library A, eight of the ten sequenced peptide dendrimers incorporated a glutamate at position A^8, five sequences had glutamate, and four had tryptophan at A^6. In library C, seven hits showed a serine at A^8, and seven sequences had a glutamate at A^6. The cysteine-containing ligands **A1** and **B1**–**B4** bound tightly and rapidly to cobalamin **1a**, but not to either **1b** or **1c**; this confirms the on-bead selection assays (Figure 3C). The role of the outer-layer glutamates in the kinetics of B_{12} binding was evidenced by the fact that the cationic analogue, **B1K**, which was obtained by replacing all of the glutamates in **B1** by lysine residues, was kinetically strongly impaired for cobalamin binding to the extent that complex formation could not be measured. UV–visible spectral changes were observed in all titrations that indicated thiol coordination to cobalt, and clear isosbestic behavior, which implies that only free cobalamin and the complex were present in solution (Figures 2 and 3B). Coordination of the cysteine residue to cobalt was confirmed by ^{1}H NMR spectroscopy, which showed shifts in the cobalamin signals that are typical for thiol coordination.[13] Significant displacements of the aromatic residue signals of the dendrimer confirm the spatial proximity of the dendrimer to cobalamin (Figure 3D). In addition, the hydrodynamic radius of the second generation dendrimer **B1G2**, which is derived from **B1** is reduced upon binding to aquocobalamin from 1.31 nm to 1.23 nm as determined from diffusion NMR spectroscopy; this suggests that complexation to cobalt induces packing.

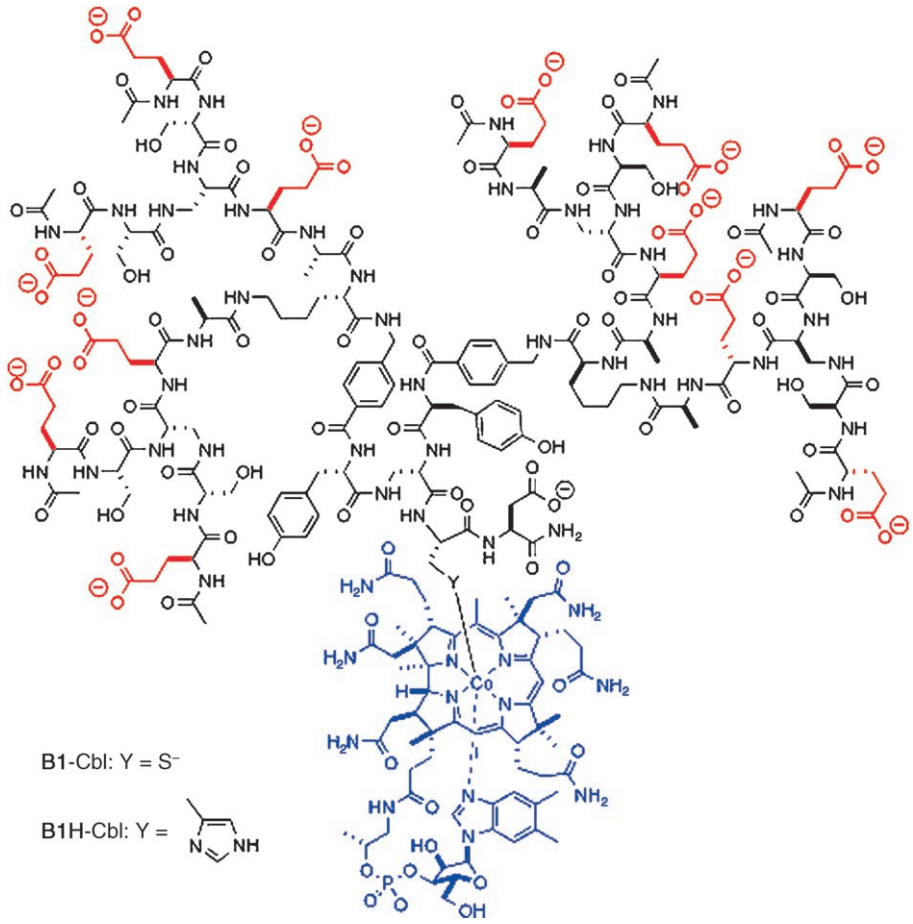

Figure 2. Structural formula of the cobalamin peptide dendrimers.

Dendrimer **C1** with histidine and glutamate residues, a positive hit from library C, bound aquocobalamin. Ligand exchange was indicated by the shifts in the UV–visible spectrum from 351 to 358 nm and from 525 to 536 nm, which is typical for imidazole coordination to cobalamins. The binding constant was $K_a = 2.2 \times 10^4\ M^{-1}$.[14] Shifts of the cobalamin signals in the ^{1}H NMR spectrum that are consistent with histidine coordination to cobalt were also observed.[15] Binding of the more hydrophobic dendrimer **C2**, which lacks glutamate residues, to aquocobalamin could not be detected in solution under the same conditions; this is in agreement with the on-bead results.[16] An additional histidine ligand, **B1H**, was obtained by replacing the coordinating cysteine in **B1** by histidine, while keeping the same outer layers. **B1H** binds aquocobalamin with four-fold higher affinity than **C1** (Table 1). Coordination of the His residue from the upper (β) side of the base-on cobalamin (Figure 2) mimics the complexation mode that was observed for the transport protein transcobalamin.[17]

The **B1**–cobalamin (Cbl) complex was remarkably stable even at strongly alkaline pH. By contrast, complexes with cys-

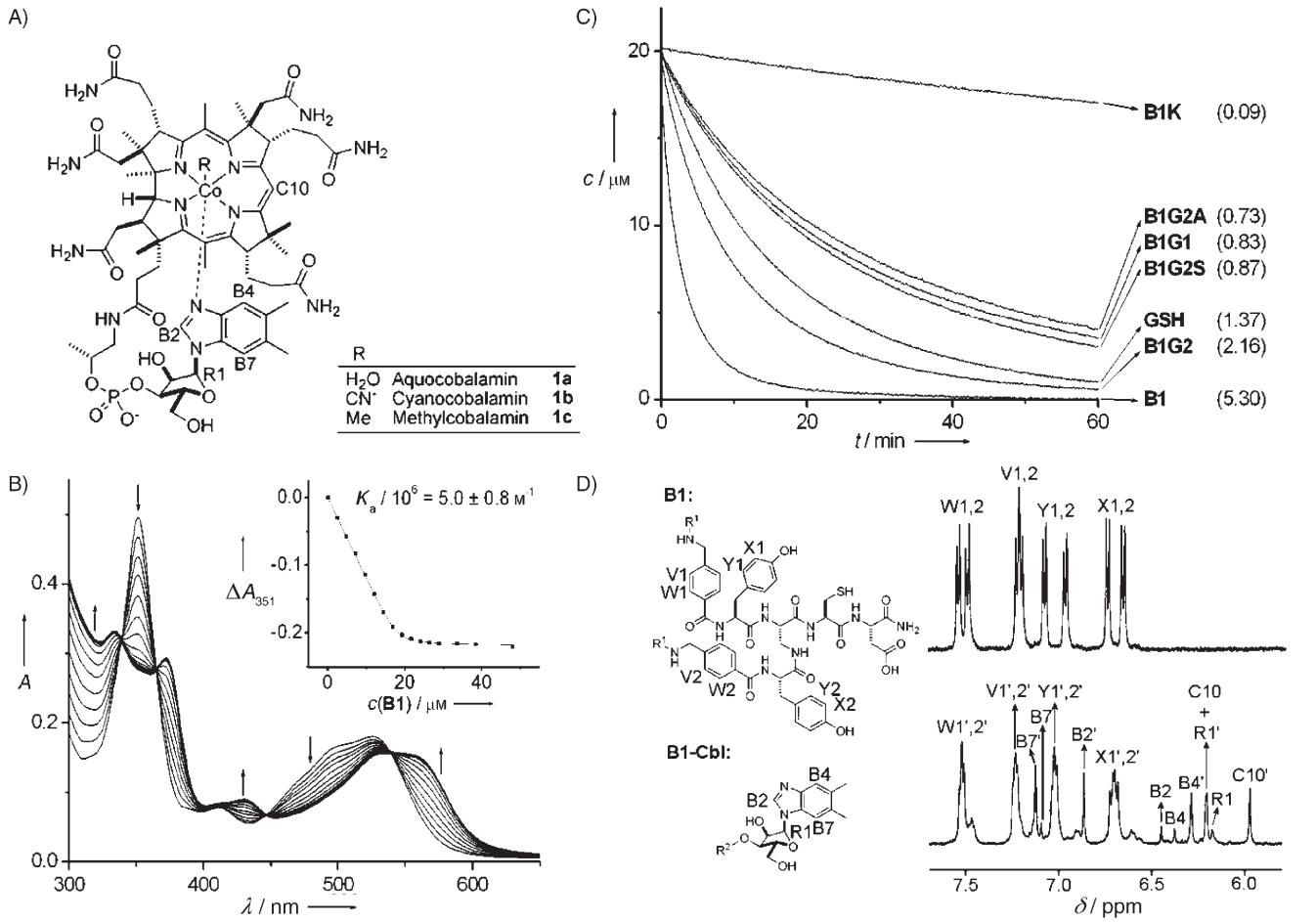

Figure 3. A) Structure of B_{12} derivatives **1a**–**c.** B) UV/Vis titration of aquocobalamin (20 μM) at pH 7.0 and 25 °C with **B1** and the change in absorption at 351 nm (insert). C) Kinetics of complex formation. Dendrimer or peptide was added to a solution of aquocobalamin, and the reaction was followed by measuring the absorption at 351 nm (initial concentrations: aquocobalamin 20 μM; ligand 50 μM) at pH 7.0, 25 °C. The *v* values are given in μM min^{-1} in parentheses, where *v* is the calculated initial rate of complexation. D) ^{1}H NMR (500 MHz) data that show the aromatic protons of peptide dendrimer **B1** (upper) and the **B1**–Cbl complex (lower), which were assigned by 2D NMR data at pD 7.0. Nonequivalent Tyr and Amb residues are present in **B1**. The prime signals belong to the complex. The unlabeled signals in the lower spectrum arise from the dimer formed by slow oxidation. An excess of aquocobalamin was added as a reference.

teine or glutathione (γ-glutamylcysteinylglycine) rapidly underwent oxidation to form disulfide and cobalt(II) (Table 2).[18,19] Investigation of the lower-generation analogues **B1G0**, **B1G1** and **B1G2** showed that the protective binding towards B_{12} increased upon addition of the dendritic layers, and the complete dendrimer, **B1** displayed the most stabilizing binding interaction. The linear peptides **B1L1**–**B1L3**, which were obtained by replacing the branching 2,3-diaminopropanoic acid by alanine in the **B1** sequence, bound cobalamin **1a**, but did not provide the protecting effect that was observed with the dendrimer ligands (Tables 1 and 2). Ligand-exchange reactions (N_3^-, NO_2^- and CN^-) were slower with the dendrimer complex **B1**–Cbl than with glutathionylcobalamin; this further evidences the shielding effect of the dendrimer towards the cysteine-bound cobalt center (Table 3). The dendrimer practically inhibited the N_3^- exchange even with a large excess of azide. The shielding effect could also be observed with nitrite and the less bulky cyanide anion, which has a very high affinity for Co^{III} ($k_a > 10^{12}$ M^{-1}). The histidine dendrimer **B1H** reduced the exchange with cyanide by a factor of six when compared to imidazole; this shows that the outer layers of the dendrimer also protect the Co^{III} that is bound to an imidazole residue. Interestingly, the shielding effect of **B1H** for the reaction with CN^- and N_3^- is comparable to the shielding that is provided by the transport protein transcobalamin.[14]

In summary, dendritic ligands for cobalamin were identified from a combinatorial peptide dendrimer library that features metal-coordinating residues at the core, and a polyanionic shell of glutamates in the outer layers. The dendritic shell provided a large kinetic effect that was evidenced by the stabilization of thiolato complexes for redox reactions, and the slower exchange rates for complexes with both cysteine and histidine dendrimers. The peptide dendrimer mimics the binding mode and kinetic features of the vitamin B_{12} transporter without, however, rivaling the protein in terms of cofactor-binding affinity (for transcobalamin $K \sim 10^{10}$ M^{-1} [2d]). The dendritic ligand is probably not organized enough to bind the corrin ring as tightly as the natural protein does.

Table 1. Synthesis of dendrimers and peptides and binding data to aquocobalamin **1a**. *B* = (*S*)-2,3-diaminopropanoic acid. X = 4-(aminomethyl)benzoic acid. X′ = β-alanine. X″ = 4-hydroxyproline. Ac = acetyl. Sequences as primary amides at the C terminus. Branching diamino acids are indicated in italics.

No	Sequences	Yield [%]	Yield [mg]	MS ligand found/calcd	$K_a/10^6$ [M^{-1}]	MS complex found/calcd
A1	$(AcET)_8(BWT)_4(BRS)_2BCL$	11	25.4	4649/4647	2.5 ± 0.4	5978/5975
B1	$(AcES)_8(BEA)_4(KXY)_2BCD$	16	33.0	4381/4379	5.0 ± 0.8	5710/5707
B2	$(AcEL)_8(BEA)_4(BX'I)_2BCD$	4	7.8	4282/4279	2.7 ± 0.3	5609/5607
B3	$(AcEX)_8(KEY)_4(BXT)_2BCD$	2	4.4	5100/5097[a]	6.7 ± 1.1	6405/6404
B4	$(AcEL)_8(BEY)_4(BSI)_2BCD$	3	6.0	4766/4763	4.2 ± 0.6	6094/6092
C1	$(AcSG)_8(BEY)_4(BHT)_2BRA$	7	12.5	3981/3982	0.022 ± 0.002	5309/5309
C2	$(AcSL)_8(BIP)_4(BHX'')_2BRA$	4	8.1	4128/4126	–[b]	–[b]
B1H	$(AcES)_8(BEA)_4(KXY)_2BHD$	12	24.0	4415/4414	0.083 ± 0.011	5744/5741
B1K	$(AcKS)_8(BKA)_4(KXY)_2BCL$	14	36.3	4369/4365	–[c]	5696/5693
B1G0	AcCD	36	19.1	278/278	2.7 ± 0.3	1605/1605
B1G1	$(AcXY)_2BCD$	40	18.6	997/997	3.6 ± 0.5	2327/2326
B1G2	$(AcEA)_4(KXY)_2BCD$	39	38.0	2138/2138	4.8 ± 0.9	3468/3466
B1G2A	$(AcA)_4(KXY)_2BCD$	28	20.9	1623/1623	5.3 ± 1.0	2953/2949
B1G2S	$(AcSA)_4(KXY)_2BCD$	34	30.4	1993/1993	5.2 ± 1.0	3300/3297
B1L1	AcXYACD	40	32.4	645/644	7.0 ± 1.7	1972/1972
B1L2	AcEAAXYACD	54	23.2	938/938	5.6 ± 0.8	2243/2243
B1L3	AcESAEAAXYACD	48	26.4	1203/1203	3.5 ± 0.4	2532/2530
GSH	Glutathione	–[d]	–[d]	307/307	1.5 ± 0.2	1636/1635

[a] $[M+Na]^+$. [b] No binding to **1a**. [c] UV/Vis titration was not carried out due to slow equilibration (Figure 3C). [d] Commercially available.

Table 2. Summary of stabilities of thiolato complexes at different pHs: + indicates unchanged UV/Vis spectra of aquocobalamin–thiol complex (similar to Figure 3B) after 2 h; − indicates that under the same conditions, UV/Vis spectra that show the presence of cobalt(II) were obtained; experiments were carried out under air-free conditions at 25 °C in HEPES (pH 7.0 and 8.0) and borate buffer (pH 9.0 and 10.0) 20 mM.

Ligand	pH 7.0	8.0	9.0	10.0
B1	+	+	+	+
B1G2	+	+	+	+
B1G1	+	−	−	−
B1G0	+	−	−	−
B1G2A	+	−	−	−
B1G2S	+	−	−	−
B1L3	+	+	−	−
B1L2	+	+	−	−
cysteine	−	−	−	−
glutathione	+	−	−	−

Table 3. Exchange rates of cobalamin ligand in the dendrimer complexes and transport proteins (TC = transcobalamin, IF = intrinsic factor and HC = haptocorrin).

Ligand in Cbl complex	*t* for 50% ligand exchange N_3^-	CN^-
B1	8% in 12 h (1 M)	30 min (0.1 mM)
B1H	50 min (50 mM)	3 h (0.1 mM)
glutathione	6.5 h (1 M)	12 min (0.1 mM)
imidazole	10 min (50 mM)	30 min (0.1 mM)
H_2O[a]	< 1 s (5 mM)	< 1 s (2.5 mM)
Tc[a]	30 min (50 mM)	50 min (1 mM)
IF[b]	< 5 s (2.5 mM)	20 s (0.5 mM)
HC[b]	< 10 s (2.5 mM)	1 min (0.1 mM)

Concentrations of N_3^- and CN^- are shown in parentheses; [Cbl] = 25 mM; 37 °C, pH 7.5 for proteins; 20 °C, pH 7.0–8.0 for other ligands. [a] Data from ref. [14a]. [b] Data from ref. [14b].

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Keywords: cobalt complexation · combinatorial chemistry · dendrimers · peptides · vitamin B_{12}

[1] a) A. Lombardi, F. Nastri, V. Pavone, *Chem. Rev.* **2001**, *101*, 3165–3189; b) M. M. Rosenblatt, J. Wang, K. S. Suslick, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 13140–13145; c) F. V. Cochran, S. P. Wu, W. Wang, V. Nanda, J. G. Saven, M. J. Therien, W. F. DeGrado, *J. Am. Chem. Soc.* **2005**, *127*, 1346–1347.

[2] a) K. L. Brown, *Chem. Rev.* **2005**, *105*, 2075–2149; b) *Chemistry and Biochemistry of B_{12}* (Ed.: R. Banerjee), Wiley, New York, **1999**; c) W. Buckel, C. Kratky, B. T. Golding, *Chem. Eur. J.* **2006**, *12*, 352–362; d) E. Nexø in *Vitamin B_{12} and B_{12} Proteins* (Eds. B. Kräutler, D. Arigoni, B. T. Golding), Wiley-VCH, Weinheim, **1998**, pp. 461–475.

[3] R. B. Hannak, R. Konrat, W. Schüler, B. Kräutler, M.-T. M. Auditor, D. Hilvert, *Angew. Chem.* **2002**, *114*, 3765–3768; *Angew. Chem. Int. Ed.* **2002**, *41*, 3613–3616.

[4] a) J. R. Lorsch, J. W. Szostak, *Biochemistry* **1994**, *33*, 973–982; b) D. Sussman, J. C. Nix, C. Wilson, *Nat. Struct. Biol.* **2000**, *7*, 53–57.

[5] a) G. R. Newkome, C. N. Moorefield, F. Vögtle, *Dendritic Molecules: Concepts, Synthesis Applications*, VCH, Weinheim, 2001; b) L. Crespo, G. Sanclimens, M. Pons, E. Giralt, M. Royo, F. Albericio, *Chem. Rev.* **2005**, *105*, 1663–1682; c) D. A. Tomalia, P. R. Dvornic, *Nature* **1994**, *372*, 617–618.

[6] a) T. Darbre, J.-L. Reymond, *Acc. Chem. Res.* **2006**, *39*, 925–934; b) J. Kofoed, J.-L. Reymond, *Curr. Opin. Chem. Biol.* **2005**, *9*, 656–664.

[7] a) A. Esposito, E. Delort, D. Lagnoux, F. Djojo, J.-L. Reymond, *Angew. Chem.* **2003**, *115*, 1419–1421; *Angew. Chem. Int. Ed.* **2003**, *42*, 1381–1383; b) C. Douat-Casassus, T. Darbre, J.-L. Reymond, *J. Am. Chem. Soc.* **2004**, *126*, 7817–7826; c) E. Delort, T. Darbre, J.-L. Reymond, *J. Am. Chem. Soc.* **2004**, *126*, 15642–15643; d) E. Delort, N.-Q. Nguyen-Trung, T. Darbre, J.-L. Reymond, *J. Org. Chem.* **2006**, *71*, 4468–4480; e) J. Kofoed, T. Darbre, J.-L. Reymond, *Org. Biomol. Chem.* **2006**, *4*, 3268–3281.

[8] a) E. Kolomiets, E. M. V. Johansson, O. Renaudet, T. Darbre, J.-L. Reymond, *Org. Lett.* **2007**, *9*, 1465–1468; b) E. M. V. Johansson, E. Kolomiets,

F. Rosenau, K.-E. Jaeger, T. Darbre, J.-L. Reymond, *New J. Chem.* **2007**, *31*, 1291–1299.
[9] D. Lagnoux, T. Darbre, M. L. Schmitz, J.-L. Reymond, *Chem. Eur. J.* **2005**, *11*, 3941–3950.
[10] a) S. Hecht, J. M. J. Fréchet, *Angew. Chem.* **2001**, *113*, 76–94; *Angew. Chem. Int. Ed.* **2001**, *40*, 74–91; b) M. Uyemura, T. Aida, *J. Am. Chem. Soc.* **2002**, *124*, 11392–11403; c) P. Bhyrappa, J. K. Young, J. S. Moore, K. S. Suslick, *J. Am. Chem. Soc.* **1996**, *118*, 5708–5711.
[11] a) A. Clouet, T. Darbre, J.-L. Reymond, *Angew. Chem.* **2004**, *116*, 4712–4715; *Angew. Chem. Int. Ed.* **2004**, *43*, 4612–4615; b) A. Clouet, T. Darbre, J.-L. Reymond, *Biopolymers* **2006**, *84*, 114–123.
[12] J. M. Pratt, *Inorganic Chemistry of B_{12}*, Academic Press, London, **1972**, pp. 139–147 and 217–218.
[13] a) A. M. Calafat; L. G. Marzilli, *J. Am. Chem. Soc.* **1993**, *115*, 9182–9190; L. G. Marzilli, *J. Am. Chem. Soc.* **1993**, *115*, 9182–9190; b) K. L. Brown, X. Zou, *Biochemistry* **1993**, *32*, 8421–8428.
[14] a) S. N. Fedosov, N. U. Fedosova, E. Nexø, T. E. Petersen, *J. Biol. Chem.* **2000**, *275*, 11791–11798; b) S. N. Fedosov, L. Berglund, N. U. Fedosova, E. Nexø, T. E. Petersen, *J. Biol. Chem.* **2002**, *277*, 9989–9996.
[15] L. Hannibal, S. D. Bunge, R. Van Eldik, D. W. Jacobsen, C. Kratky, K. Gruber, N. E. Brasch, *Inorg. Chem.* **2007**, *46*, 3613–3618.
[16] Dendrimer C2 coordinates the iron center in heme; this shows that the histidine residue is available for binding.
[17] J. Wuerges, G. Garau, S. Geremia, S. N. Fedosov, T. E. Petersen, L. Randaccio, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 4386–4391.
[18] a) F. Wagner, K. Bernhauer, *Ann. N. Y. Acad. Sci.* **1964**, *112*, 580–589; b) E. Pezacka, R. Green, D. W. Jacobsen, *Biochem. Biophys. Res. Commun.* **1990**, *169*, 443–450.
[19] For glutathione and dipeptide–cobalamin complexes, see: R. K. Suto, N. E. Brasch, O. P. Anderson, R. G. Finke, *Inorg. Chem.* **2001**, *40*, 2686–2692.

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