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Trifluoracetic Acid-Assisted Crystallization of Vitamin B_{12} Results in Protonation of the Phosphate Group of the Nucleotide Loop: Insight into the Influence of Crystal Packing Forces on Vitamin B_{12} Structures

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In the course of experiments concerning our ongoing project on the synthesis of vitamin B_{12} (cyanocobalamin, CNCbl) bioconjugates for drug-delivery purposes, we observed the formation of well-shaped red parallelepipeds from a concentrated aqueous solution of the HPLC-purified vitamin. The X-ray structural investigation (MoK_α) at 98 K on these crystals revealed a CNCbl-TFA salt of formula [CNCbl(H)](TFAc)·$14H_2O$ (**1**, where TFA = trifluoracetic acid; $TFAc^-$ = trifluoracetate anion), in which a proton transfer from the trifluoracetic acid to the phosphate-O4P oxygen atoms is observed. **1** crystallizes in the standard orthorhombic $P2_12_12_1$ space group, a = 16.069(2) Å, b = 20.818(2) Å, c = 24.081(2) Å, Z = 4. The final full-matrix least-squares refinements on F^2 converged with R_1 = 4.1% for the 18957 significant reflections, a very low crystallographic residual for cobalamins, which facilitated the analysis of the extensive network of hydrogen bonds within the lattice. To the best of our knowledge, this is the first cobalamin structure to show protonation of the phosphate group of the cobalamin nucleotide loop. In this work, the crystal structure of **1** is analyzed and compared to other CNCbls reported in the literature, namely, CNCbl·3PrOH·$12H_2O$ (**2**, PrOH = propyl alcohol), CNCbl·acetone·$20H_2O$ (**3**), CNCbl·2LiCl·$10.2H_2O$ (**4**), and CNCbl·2KCl·$10.6H_2O$ (**5**). The analysis confirmed that protonation of the phosphate leaves the major CNCbl structural parameters unaffected, so that **1** can be considered an "unmodified" Cbl solvate. However, comparison between **1**−**5** led to interesting findings. In fact, although the cobalt(III) coordination sphere in **1**−**5** is similar, significant differences could be noted in the upward fold angle of the corrin macrocycle, a parameter commonly related to the steric hindrance of the axial lower "α" nucleotide-base and the electronic *trans influence* of the upper "β" substituent. This suggests that crystal-packing forces may influence the corrin deformation as well. Herein we explore, on the basis of the newly acquired structure and reported crystallographic data, whether the incongruities among **1**−**5** have to be attributed to random crystal packing effects or if it is possible to associate them with specific crystal packing (*clusters*).

Introduction

The development of vitamin B_{12} (cyanocobalamin; CNCbl) bioconjugates for delivery and/or targeting of imaging/therapeutic agents is an area currently undergoing considerable growth.[1−4]

Our own contribution to this field spans from probe design[4b] and oral peptide/protein delivery[4a] to investigations on the implications of CNCbl modifications on biological activity.[5] Utilizing molecular dynamics methodology to predict biological outcomes, we have also recently shown the great potential of computational methods as a screening/design tool for CNCbl-bioconjugates.[5b]

Given that theoretical simulations require structural data as reference models,[5b,6] a complete understanding of the

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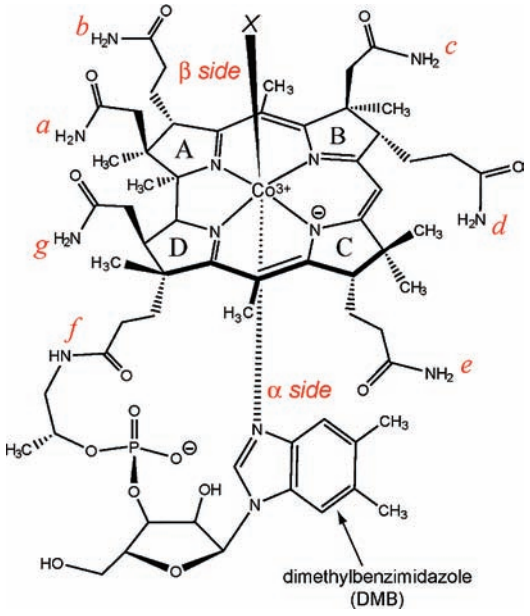
structural "makeup" of cobalamins (Cbls) and the structure of CNCbl-proteins and their receptors is warranted. Highlights in such investigations[7−10] are the publication by Sukumar et al.[8] and Randaccio et al.[9] of two of the three CNCbl-transport proteins, intrinsic factor (IF) and trans-cobalamin II (TC), respectively, in their holo form (i.e., IF-CNCbl and TC-CNCbl complexes). In 2010, a detailed picture of the interactions between the IF-CNCbl complex and the cubilin receptor-IF binding region (CUB_{5-8}) was reported by Andersen et al.[10]

Despite these achievements with systems of such structural complexity, cobalamin-crystallography itself still holds challenges. Significant progress has been made in this field since the original crystal structure of CNCbl and coenzyme B_{12} (adenosylcobalamin; AdoCbl) were reported by Hodgkin and collaborators in the early 1950s−1960s,[11] thanks in large part to the wider availability/use of synchrotron radiation and improvements in crystallization techniques.[12] Considerable efforts have been devoted to the crystallization of biologically relevant Cbls[13−28] as well as to the validation of the solid state approach for the study of these systems, successfully challenging structural results with in-solution NMR[16,17,29−32] and/or EXAFS[33] analysis.

Scheme 1. Molecular Structure of XCbls[a]



[a] The central cobalt(III) atom is six-coordinated, with the equatorial positions filled by the nitrogen atoms of the corrin macrocycle. The (conventionally) lower, "α"-axial site is occupied by an imidazole-nitrogen atom from a 5′,6′-dimethylbenzimidazole (DMB) base while the upper, "*β*"-axial site can be occupied by various *X* groups. The corrin ring incorporates seven amide side chains, three acetamides (*a*, *c*, *g*) and four propionamides (*b*, *d*, *e*, *f*). The four pyrrole rings are usually indicated as A, B, C, and D, as shown.

While structures of Cbl-(small molecule) conjugates are still rare (see for example the work of Alberto et al.[34,35a−35e]) dozen of XCbls (i.e., vitamin B_{12} analogues, see Scheme 1) structures have been reported, a discrete number of which show medium to high resolution (see Table 1).

Given their biological importance,[12,36,37] the strength of the two axial bonds in Cbl systems has been extensively investigated, with attempts made to correlate axial fragments

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Table 1. Structurally Characterized XCbls[a]

year	CSD refcode	*X* (bound atom)	crystal formula	R_1 (%)	λ (cm^{-1})[b], T[c]	reference
2010		cyano (C)	CNCbl·TFA·$14H_2O$	4.10	MoK_α, 98 K	This work
2007	GIBMOU	cyano (C)	CNCbl·3PrOH·$12H_2O$	3.32	MoK_α, 100 K	13
1994	HEWPUU	cyano (C)	CNCbl·acetone·$20H_2O$	8.24	MoK_α, 88 K	14
2000	WIKXUJ	cyano (C)	CNCbl·2LiCl·$10.2H_2O$	4.35	0.8, 100 K	15
2000	WIKYAQ	cyano (C)	CNCbl·KCl·$10.6H_2O$	7.33	0.8, 100 K	15
1985	CURWOB	methyl (C)	MeCbl·acetone·$40H_2O$	14.60	MoK_α, RT	16
2000	WIKXOD	methyl (C)	MeCbl·$18.1H_2O$	8.34	0.8, 100 K	15
1998	NAWSUZ	trifluoromethyl (C)	CF_3Cbl·2acetone·$6H_2O$	9.66	MoK_α, RT	48
1999	FIQXEI	difluoromethyl (C)	CHF_2Cbl·$17H_2O$	7.16	MoK_α, 120 K	56
2009	YUCCAB	ethyl (C)	EtCbl·$17H_2O$	4.62	0.78468, 100 K	38
2009	YUCCEF	*n*-buthyl (C)	*n*BuCbl·$21H_2O$	4.69	0.78468, 100 K	38
2004	ISUKAI	*i*-amyl (C)	*i*AmCbl·0.39acetone·$12.65H_2O$	4.26	MoK_α, 123 K	57
1985	CUVCIF	*R*-2,3-dihydroxypropyl (C)	[R-$(OH)_2$PrCbl]·$15H_2O$	8.50	MoK_α, 98 K	58
1985	CUVCOL	*S*-2,3-dihydroxypropyl (C)	[S-$(OH)_2$PrCbl]·$15H_2O$	15.10	MoK_α, 98 K	58
2005	FEZJAW	vynil (C)	CH_2CHCbl·$18H_2O$	8.56	MoK_α, 163 K	59
2003	GUZDOU	chlorovynil (C)	*cis*-CClHCHCbl·$17H_2O$	10.12	MoK_α, 153 K	60
1991	VIPJAF	adenylpropyl (C)	AdePrCbl·acetone·$40H_2O$	7.90	MoK_α, 163 K	17
1968	DADCBL	adenosyl (C)	AdoCbl·$17H_2O$	13.20	MoK_α, RT	18a
2004	ATETUO	adenosyl (C)	AdoCbl·1.25acetone·$8.25H_2O$	6.61	0.737, 100 K	18c
1998	VOKNIT	α-ribofuranosyladinyl (C)	(α-ribo)AdoCbl·MeCN·$19H_2O$	7.08	MoK_α, RT	19
2005	FEXSIL	adenosylmethyl (C)	AdoMeCbl·3acetone·$17.5H_2O$	5.79	0.8431, 103 K	20
2003	EJADOI	nitro (N)	NO_2Cbl·acetone·$12.25H_2O$	7.16	MoK_α, 123 K	21
2003	HUSPAM	nitro (N)	NO_2Cbl·2LiCl·$8H_2O$	10.43	0.8, 100 K	22
2003	HUSPEQ	nitro (N)	NO_2Cbl·NaCl·$6.7H_2O$	12.07	0.8, 100 K	22
2007	SIHJOJ	nitrosyl (N)	NOCbl· $15H_2O$	9.58	0.8265, 100 K	23
1998	GIZYUJ	azido (N)	N_3Cbl·2LiCl·$9.3H_2O$	5.13	0.8, 100 K	61
2003	EJADAU	isothiocyanato (N)	SCNCbl·$15H_2O$	8.75	MoK_α, 173 K	21
2003	HUSPIU	isothiocyanato (N,S)	SCNCbl·1.7acetone·$9H_2O$	7.14	0.71, 100 K	22
2007	GIFYEA	imidazolato (*N* = N3)	ImCbl·$22H_2O$	8.11	MoK_α, RT	24
2007	GIFYAW	hystine-hystidinato (*N* = N3)	[(HisCbl$^+$)Cl^-](HisCbl)·$20H_2O$	7.34	MoK_α, RT	24
2000	XANNEG	hydroxo (O)	OHCbl·$13.7H_2O$	6.38	0.8, 100 K	25
1995	SUNYEF	aquo (O)	(H_2OCbl)ClO_4·$22.5H_2O$	4.54	0.65, RT	33
2004	INIZUA	di-*i*-propylphosphito (P)	(*i*PrO)$_2$OPCbl·3.48acetone·$7.56H_2O$	4.10	MoK_α, 123 K	62
1999	DEMSIX	sulfito (S)	NH_4(SO_3Cbl)·$13.65H_2O$	5.35	0.8, 100 K	26
2002	NOJKIG	sulfito (S)	NH_4(SO_3Cbl)·glycerol·$12H_2O$	6.03	0.8, 100 K	27
2003	EJADIC	thiosulfato (S)	Na(S_2O_3Cbl)·acetone·$15H_2O$	5.89	MoK_α, 123 K	21
1999	DEMTUK	thiourea (S)	[$(NH_2)_2$CSCbl]Cl·$10.4H_2O$	11.29	0.8, 100 K	26
2002	NOJNOP	thiourea (S)	[$(NH_2)_2$CSCbl]PF_6·$13.3H_2O$	7.32	0.8, 100 K	27
2001	XIQSEV	γ-glutamylcysteinyl (S)	γ-GluCysCbl·$22H_2O$	10.88	MoK_α, 168 K	28
2006	MESSOT	*n*-acetyl-L-cysteinyl (S)	Na(*n*ACysCbl)·$18H_2O$	10.30	0.81798, 100 K	46
1998	GIZYOD	chloro (Cl)	ClCbl·2LiCl·$7.5H_2O$	4.86	0.8, 100 K	61
2003	EJADEY	selenocyanato (Se)	NCSeCbl·$15H_2O$	7.46	MoK_α, 123 K	21
2003	HUSPOA	selenocyanato (Se)	NCSeCbl·acetone·$11H_2O$	8.76	0.71, 100 K	22

[a] CSD database search November 2010. [b] MoK_α = 0.71073 cm^{-1}. Numerically specified wavelengths refer to synchrotron radiation. [c] RT = room temperature (298 K).

with corrin deformation,[12,36] with the ultimate goal to understand their role in enzymatic activity.[14,20] Although the bulk of the dimethylbenzimidazole base (α-axial group) has been determined to be a relevant factor for the corrin deformation,[14] the role of the axial β-substituent is not uniquely defined. The existence of an electronic "trans influence" has been postulated on the basis of crystallographic data for XCbls,[12,36,38−40] which indirectly affects the corrin folding by acting on the Co−N bond length. However, the X groups may exercise a direct bulky effect as well, complicating the correlation. With the observation of a so-called "packing invariance"[12b] among Cbls crystal structures contributing to the idea that crystal packing forces are not likely to influence the structural chemistry of Cbls, apparent anomalies in the observed trends have been mostly attributed to crystallographic inaccuracy.[12] On the basis of recent density functional theory (DFT) studies however, the assumption of the innocence of crystal packing needs to be re-examined, at least for cobalamins with "bulky" β-axial substituents (e.g., AdoCbl).[41]

Multiple structural determinations are currently available for a variety of Cbls such as CN-,[13−15] CH_3-,[15,16] Ado-,[18] NO_2-, SCN-, SeCN-,[21,22] SO_3-, and thiourea-[26,27] cobalamin (see Supporting Information, Table S1). Among these, "modern" CNCbl structures, namely, CNCbl·3PrOH·$12H_2O$ (**2**, PrOH = propyl alcohol),[13] CNCbl·acetone·$20H_2O$ (**3**),[14] CNCbl·2LiCl·$10.2H_2O$ (**4**), and CNCbl·2KCl·$10.6H_2O$ (**5**),[15] appear to offer sufficiently accurate crystallographic information to look closely at this area from a structural point of view and question the latest computational findings on the substantial irrelevance of crystal packing effects for Cbls with "small" β-axial substituents.[41] From our perspective, we have been inspired to do so after noticing the rapid and facile formation of a new CNCbl crystalline form, obtained from an acidic water solution containing the HPLC-purified vitamin. Single crystal X-ray diffraction revealed that trifluoroacetic acid (TFA) from the HPLC purification method was being

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incorporated into the crystalline sample, to yield an unprecedented CNCbl salt of formula [CNCbl(H)](TFAc)·14H_2O (**1**, ($TFAc^-$ = trifluoroacetate), with the Cbl protonation occurring at the phosphate group. We were subsequently able to grow the crystals by direct addition of 0.1% TFA to concentrated CNCbl solutions, repeatedly obtaining high quality single crystals in less than 24 h at 4 °C. Upon investigating the influence of the protonation on the $[CNCbl(H)]^+$ molecule by comparing **1** with **2**−**5**, we discovered significant differences among these structures, which cannot be exclusively attributed to experimental errors. The reliability of this set of data in terms of crystallographic resolution and the variability of crystallographic settings among **1**−**5** offered up the possibility of investigating the role of crystal packing forces in the solid-state chemistry of CNCbl. The results of our analysis are presented herein, with a revised general analysis of the four main crystal packing typologies[12] within Cbls structures put forward.

Experimental Section

Materials and Methods. Acetonitrile (MeCN, 99.8%, Sigma) and trifluoroacetic acid (TFA, 99%, Aldrich) were purchased and used as received. Cyanocobalamin (CNCbl) was purchased from Sigma Aldrich (stated purity by manufacturer ∼99%). Water was distilled and deionized to 18.6 MΩ using a Barnstead Nano Diamond ultrapurifier. Reverse phase high-pressure liquid chromatography (RP-HPLC) was performed using an Agilent 1100 or 1200. A C_{18} semiprep column (9.4 mm × 250 mm) was utilized with a flow rate of 1 mL/min. Detection was carried out by UV monitoring at 254 nm. Elution was performed using 0.1% TFA spiked water (A) and MeCN (B) as solvents in isocratic mode (20% B) over 8 min.

Crystallization of [CNCbl(H)](TFAc)·14H_2O (1). Thirty milligrams of CNCbl were dissolved in 3 mL of water and purified via RP-HPLC. Red fractions confirmed to be CNCbl by ^{1}HNMR were combined and dried in vacuo. This dried sample was redissolved in 500 μL of water, and the solution (noted as pH = 2) was left at 4 °C. Red parallelepiped shaped crystals of **1**, suitable for X-ray structural investigation, grew overnight. This result could subsequently be achieved, bypassing the HPLC purification, by directly dissolving CNCbl in 0.1% TFA.

Crystal Structure Determination and Refinement. X-ray crystallographic data for **1** were collected with a Bruker-AXS SMART CCD diffractometer at 98 K using graphite monochromated MoKα radiation (λ = 0.71073 Å). The crystals where coated with Paraton oil to prevent solvent loss, attached to a glass fiber and quickly transferred under the cold nitrogen stream of the diffractometer. For data collection and integration, the Bruker SMART[42] and SAINT[43] softwares were employed. Data were scaled using SADABS,[44] and no absorption correction was applied. The structure was solved by direct methods and subsequently completed by Fourier recycling using the SHELXTL[45] software packages and refined by the full-matrix least-squares refinements based on F^2 with all the observed reflections (Friedel opposites not merged).

All non-hydrogen atoms were refined anisotropically and all the cobalamin-hydrogen atoms were set in calculated positions and refined using a riding model. The hydrogen atoms on the water molecules of crystallization were located on the ΔF map and then refined with restraints, with thermal factors fixed at 0.05 $Å^2$. The hydrogen atom on the phosphate group (O4P) was located on the ΔF map as well and refined without restraints. Flack's analysis supports the absolute configuration of the model. Crystal data for **1** are summarized in Table 2. CCDC reference number 791268.

Table 2. Summary of the Crystal Data for [CNCbl(H)](TFAc)·14H_2O (**1**)

	1
formula	$C_{65}H_{117}CoF_3N_{14}O_{30}P$
M_r	1721.63
crystal system	orthorhombic
space group	$P2_12_12_1$
a/Å	16.069(2)
b/Å	20.818(2)
c/Å	24.081(2)
V/$Å^3$	3626(3)
Z	4
D_c/g cm^{-3}	1.420
T/K	98(2)
F(000)	3656
μ(Mo−Kα)/mm^{-1}	0.330
refl. collected	20116
refl. indep.	7432
refl. obs. [$I > 2\sigma(I)$]	4513
no. param.	1120
$R_1{}^a$ [$I > 2\sigma(I)$] (all)	0.0409 (0.0444)
$wR_2{}^b$ [$I > 2\sigma(I)$] (all)	0.1040 (0.1084)
goodness-of-fit on F^2	1.105
abs. struct. param.	−0.004(8)
$\Delta\rho_{max,\ min}$/e $Å^{-3}$	0.690 and −0.361

^{a} $R_1 = \sum||F_o| - |F_c||/\sum|F_o|$. ^{b} $wR_2 = \{\sum w(F_o{}^2 - F_c{}^2)^2/\sum w(F_o{}^2)^2\}^{1/2}$ and $w = 1/[\sigma^2(F_o{}^2)+(mP)^2+nP]$ with $P = (F_o{}^2 + 2F_c{}^2)/3$, $m = 0.0612$ and n = 2.9745.

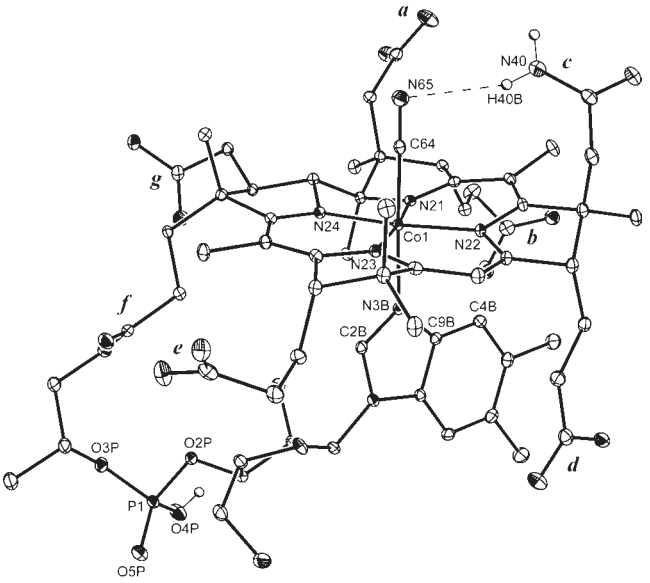

Figure 1. ORTEP plot (30% probability level) of the $[CNCbl(H)]^+$ molecule in **1** with selected atomic labels. Only selected hydrogen atoms are shown for clarity.

Results and Discussion

Crystal Structure of [CNCbl(H)](TFAc)·14H_2O (1). The ionic adduct **1** [CNCbl·TFA] crystallizes in the orthorhombic space group $P2_12_12_1$, incorporating 14 ordered water molecule of crystallization with full occupancy per formula unit. A $CNCbl(H)^+$ cation (in the "base-on" conformation[12]) and

(42) *SMART, Data Collection Software*, version 4.050; Siemens Analytical Instruments Inc.: Madison, WI, 1996.
(43) *SAINT, Data Reduction Software*, version 4.050; Siemens Analytical Instruments Inc.: Madison, WI, 1996.
(44) Sheldrick, G. M. *SADABS*; University of Göttingen: Göttingen, Germany, 1996.
(45) *SHELXTL PC*; Siemens Analytical X-Ray Instruments Inc.: Madison, WI, 1993.
(46) Suarez-Moreira, E.; Hannibal, L.; Smith, C. A.; Chavez, R. A.; Jacobsen, D. W.; Brasch, N. E. *Dalton Trans.* **2006**, 5269.

Table 3. Selected Bond Distances (Å) and Angles (deg) for **1**−**5**

	CNCbl·TFAc (**1**)	CNCbl (**2**) [36]	CNCbl (**3**) [22]	CNCbl·2LiCl (**4**) [33]	CNCbl·KCl (**5**) [33]
Co1−C64	1.876(2)	1.874(2)	1.86(1)	1.886(4)	1.868(8)
Co1−N3B	2.028(2)	2.048(1)	2.01(1)	2.041(3)	2.029(6)
Co1−N21	1.885(2)	1.888(1)	1.875(8)	1.881(3)	1.864(5)
Co1−N22	1.909(2)	1.922(1)	1.908(8)	1.911(3)	1.904(5)
Co1−N23	1.912(2)	1.922(1)	1.917(9)	1.920(3)	1.906(5)
Co1−N24	1.896(2)	1.902(1)	1.875(8)	1.883(3)	1.896(5)
C64−Co1−N3B	176.25(8)	176.97(4)	176.4(4)	178.0(1)	174.7(2)
Co1−C64−N65	179.26(2)	178.70(9)	179(1)	178.2(3)	175.6(6)
Co1−N3B-C2B	122.5(1)	121.45(8)	123.3(8)	123.2(2)	122.8(4)
Co1−N3B-C9B	132.2(1)	132.71(9)	133.0(7)	131.9(2)	132.5(4)
P1−O2P	1.585(2)	1.608(1)	1.585(8)	1.626(3)	1.607(5)
P1−O3P	1.580(2)	1.582(1)	1.574(7)	1.575(2)	1.492(6)
P1−O4P	1.530(2)	1.497(1)	1.505(9)	1.487(3)	1.530(2)
P1−O5P	1.470(2)	1.495(1)	1.484(9)	1.489(3)	1.495(5)

a $TFAc^-$ anion (from the preparative HPLC purification), are the two components of this molecular salt. A perspective drawing of the cationic $[CNCbl(H)]^+$ molecule in **1**, showing the proton site as O4P, is depicted in Figure 1, while selected bond lengths and angles are collected in Table 3. The *c* side chain points toward the *β*-axial cyano group to form a weak intramolecular hydrogen bond[12a] [N40−H40B···N65 2.4 Å; N40−H40B-N65 151°] while the amide functionality of the *d* side chain interacts with the benzyl ring of the DMB base via intramolecular *π*−*π* stacking [dihedral angle between the two mean planes = 7.6°; interplanar distance = 3.6 Å]. Intermolecular hydrogen bond interactions, involving the *a*, *b*, *d*, *e*, and *g* side chains, are observed as well, which directly connect each $[CNCbl(H)]^+$ with other three neighboring molecules in the *ab* plane [$a_1 \rightarrow d_2$; $g_1 \rightarrow b_2$; $b_1 \rightarrow g_3$; $d_1 \rightarrow a_3$; $e_1 \rightarrow a_4$]. Typically, the carbonyl oxygen atom is the H-bond acceptor in chains *a* and *b*, while the amide nitrogen is the donor for chains *d*, *e* and *g* (Supporting Information, Table S2).

Unlike most reported XCbl structures, **1** is characterized by a well-defined pattern of water molecule of crystallization and anions within the lattice, with no difference observed in the level of order between the pocket and the channel regions.[12a] Because of genuine novelty of this structure in the Cbl family, hydrogen bonds involving the phosphate group and the $TFAc^-$ anion are illustrated in Figure 2; a more detailed picture of the hydrogen bonding interactions in **1** is given in the Supporting Information, Figure S1, Table S2. It has been proposed[17] that ordered networks may be driven by the absence of lattice water molecules hydrogen-bonded only to other water molecules. However, in some of the more recent, better resolved XCbl structures, defined solvent positions are observed in the absence of such conditions.[38] Supporting Information, Figure S1 shows there is a water molecule (O10w) also in **1** participating in hydrogen-bonding exclusively with other water molecules.

In the extended hydrogen bond network in **1** the ribose and phosphate (O4P−H) hydroxyl groups are directed toward water molecules, acting as hydrogen bond donors (Supporting Information, Table S3).

Discussion on the Structural Consequences of the Protonation of the Phosphate Moiety in 1. As illustrated in Figure 3, the classical phosphate-water molecules motif found in the majority of the known XCbl structures, comprising **2**−**5**, can be found in **1** also. Since the protonation site is far away from the corrin, the cyano group and the DMB base, **1** can still be considered an "unmodified" Cbl solvate. However, slight changes in the geometry of the protonated phosphate group

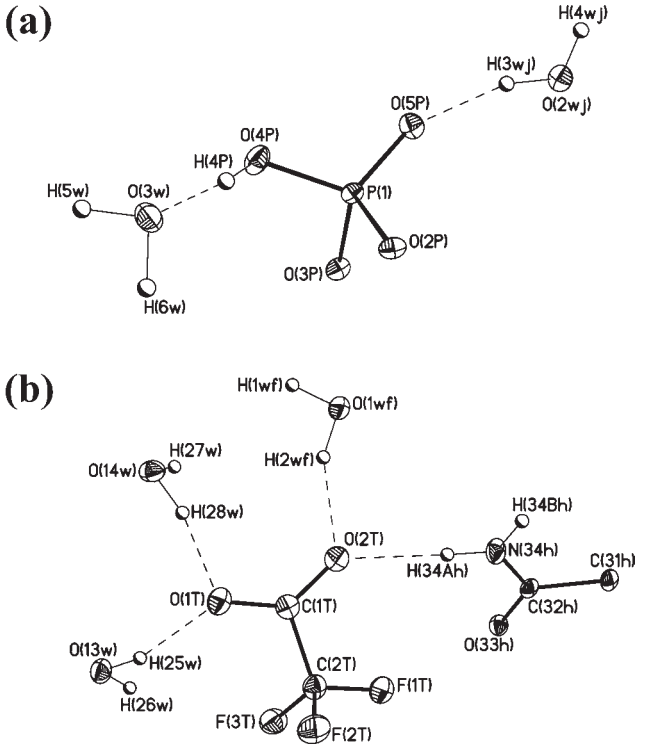

Figure 2. Hydrogen bonds (dashed lines) involving (a) the protonated phosphate group and (b) the trifluoracetate anion in **1** (thermal ellipsoid drawn at the 30% probability level). P−O distances are collected in Table 3. C−O distances in the $TFAc^-$ anion: C(1T)−O(1T) 1.235(3) Å, C(1T)−O(2T) 1.247(3) Å. Selected H-bonds details: O(4P)−H(4P)···O(3w) 2.483(3) Å; O(2wj)−(H3wj)···(O5P) 2.711(2) Å; O(13w)−H(25w)···(O1T) 2.914(3) Å; O(14w)−H(28w)···(O1T) 2.697(3) Å; O(1wf)−H(2wf)···(O2T) 2.785(3) Å; N(34 h)−H(34Ah)···(O2T) 2.957(3) Å [symmetry operation used to generate equivalent atoms: (f) $-x+1$, $y+1/2$, $-z+3/2$; (h) $-x+1/2$, $-y+2$, $z-1/2$; (j) $x+1/2$, $-y+3/2$, $-z+1$].

(in **1**) with respect to the unprotonated forms (**2**−**5**) can be noted, with the protonation freezing the P1−O4P and P1−O5P distances to values consistent with a P−OH and P═O bond, respectively, thus eliminating the possibility of the resonance occasionally observed in classic $P-O^-/P{=}O$ systems (see, for instance, **2** and **4**)

The most important finding herein is the phosphate protonation itself. It must be noted that the cationic cyanocobalamin molecule in **1** is unprecedented. In fact, the ionic XCbls reported so far (see Table 1) typically possess neutral (H_2O,[33] histidine,[24] thiourea[26,27]) or dianionic (SO_3^{2-},[26,27] $S_2O_3^{2-}$,[21] *n*-acetyl-L-cysteinyl[46]) X groups.

The incorporation of $TFAc^-$ counterions in the crystal lattice of **1**, necessary to ensure the electroneutrality of the solid phase, is interesting as well. The presence of this anion in Cbls structures has rarely been observed so far, with notable examples being the structures of the two cyano-bridged CNCbl-cisplatin (or cisplatin derivative) conjugates [*cis*-$(NH_3)_2$ClPt-CNCbl]$^{+}$[35b] and [enClPt-CNCbl]$^+$ (en = ethylenediamine).[35c] Of note, in the purification of both the CNCbl-Pt conjugates, RP-HPLC was conducted using TFA-spiked mobile phases.

$TFAc^-$ anions have already been shown to stabilize the crystal packing of diverse expanded porphyrins systems.[47] In **1**, the anion is involved, as acceptor, in a total of four hydrogen bonds (NH···O, OH···O, see Supporting Information, Figure 2 and Table S3), suggesting its presence may play a crucial role for the observed extensive crystallographic order within the structure. If this is the case, this method may represent a facile route to crystallizing Cbls.

Comparison between the Structures 1−5. The crystal structure analyses of **1**−**5** were all carried out at low temperatures (88−100K), with medium to high crystallographic resolution obtained in each case (crystallographic residual in the range 3.3−8.2%, see Table 1), which in principle offers the possibility of generating significant compare/contrast conclusions. This set is particularly interesting also because of the variability in terms of solvent nature and ionic content among these structures, as illustrated below.

3 was reported by Kräutler and Kratky in 1994 and represented the first significant "modern" redetermination of the crystal structure of CNCbl.[14] Crystals with composition CNCbl·acetone·$20H_2O$ were grown using acetone as a precipitating agent. A diffuse disorder characterizes the solvent region in **3**, as was observed in the original Hodgkin structure (so-called "wet" vitamin B_{12}[11e]), which greatly influenced the final R-index (8.2%). Crystals of the water-propanol solvate of the vitamin, with formula CNCbl·3PrOH·$12H_2O$ (**2**, PrOH = propyl alcohol), were isolated by Luger et al. in 2007.[13] The crystallographic residual reported for this structure is the lowest achieved to date for cobalamins, at 3.3%, despite one of the three propanol molecules of crystallization being statistically disordered over two positions. A fully ordered pattern of water molecules of crystallization is found within the lattice of **2**, as observed for **1**. Data for **1**−**3** were all collected using a conventional X-ray source (MoK_α). The last two CNCbl structures in the chosen set, CNCbl·2LiCl·$10.2H_2O$ (**4**) and CNCbl·KCl·$10.6H_2O$ (**5**), have been reported by Randaccio and co-workers in 2000.[15] They incorporated chloride and alkali-metal ions (Li^+ in **4** and K^+ in **5**) within the crystal lattice, thus representing a special case among the CNCbl structures **1**−**5**. In fact, because of the presence of metal ions with "coordinating" properties, such as Li^+ or K^+, **4** and **5** should be more appropriately described as three-dimensional Cbl-based polymers than as isolated Cbls structures like **1**−**3**. As reported by the authors, crystals of **4** and **5** were grown using the hanging-drop method, to promote high crystal quality.[15] Refinement of the diffraction data, collected using synchrotron radiation, gave excellent crystallographic residues (4.3% and 7.7% for **4** and **5**, respectively). Extensive metal ion-cobalamin interactions are observed in both structures [Li^+-bound sites in **4**: *a*, *d*, *e*, and *g* chains + phosphate; K^+-bound sites in **5**: *c* and *g* chains + phosphate/ribose], which decrease the conformational disorder of the flexible corrin side chains and promote a certain level of order among the water molecules of crystallization, at least in the pocket region.[12a,15]

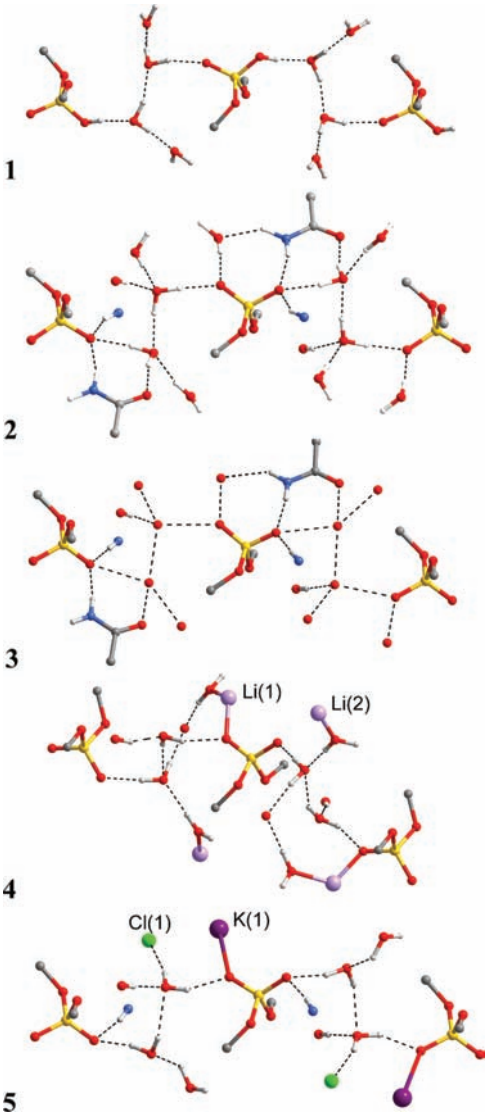

Figure 3. View of a portion of the crystal packing of **1**−**5**, showing the phosphate moiety and two crystallographically independent water molecules forming infinite step-like chains running along the *a* axis. Directly bound water molecules, amide residues, and/or ions are shown as well.

Given the reliability of these five structures (in terms of crystallographic accuracy) and the uniformity of the set (in terms of collection temperature), we believe the profound differences in the solvent nature and presence/absence of interacting charged species within the lattice of **1**−**5** would offer us the occasion to clarify (i) if the "extra" content (i.e., nature and amount of solvent or ions) can affect the "packing type"[12] of a given XCbl species or if this is basically directed by the nature (bulk, electronic properties, hydrogen bonding ability) of the

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(48) Zou, X.; Brown, K. L. *Inorg. Chim. Acta* **1998**, *267*, 305.

Table 4. Structurally Characterized CNCbls and Modified/Conjugated CNCbl[a]

	year	CSD refcode (ref)	R_1(%)	space group	a/Å	b/Å	c/Å	cluster	fold angle ϕ /deg
CNCbl·TFA (**1**)	2010	This work	4.10	$P2_12_12_1$	16.064	20.811	24.073	I	15.55(5)
CNCbl·PrOH (**2**)	2007	GIBMOU (13)	3.32	$P2_12_12_1$	15.831	22.374	25.304	II	15.9
CNCbl·acetone (**3**)	1994	HEWPUU (14)	8.24	$P2_12_12_1$	15.838	21.927	25.689	II	18.0
CNCbl·2LiCl (**4**)	2000	WIKXUJ (15)	4.35	$P2_12_12_1$	15.360	22.493	23.830	IV	18.7
CNCbl·KCl (**5**)	2000	WIKYAQ (15)	7.33	$P2_12_12_1$	16.015	21.146	23.843	I	14.1
				Side Chains Modifications					
b-acid	1984	COVDEW01 (49)	14.00	$P2_12_12_1$	14.845	17.435	16.243	N/A	21.4
C8-Me-butanamide	1997	RAFWIE (50)	5.54	$P2_12_12_1$	16.041	21.936	25.430	II	9.8
C10−Cl	1997	RIKGAT (51)	5.04	$P2_12_12_1$	16.235	21.853	26.755	II	17.7
C8-epi	1995	YUDHAG (52)	14.00	$P2_12_12_1$	14.923	17.290	32.447	none	23.8
C13-epi	1972	NEOVBT[b] (53)	15.90	$P2_12_12_1$	15.740	22.060	24.980	II	21.8
C13-epi	1996	NEOVBT01 (31)	4.04	$P2_12_12_1$	15.337	21.592	24.176	II	23.8
C2PR-nor	2006	PEFWIH (54)	7.96	$P2_12_12_1$	15.573	22.846	24.583	II or IV?	15.9
				Conjugates					
b chain	2007	CIGSAN (34a)	5.94	$P2_12_12_1$	15.957	22.302	25.692	II	18.3
d chain	2007	CIGSER (34a)	8.24	$P2_12_12_1$	15.920	22.190	25.680	II	17.8
O5′	2007	YIWJAQ (34b)	7.74	$P2_12_12_1$	15.977	22.270	26.149	II	15.2
O5′	2007	YIWJIY (34b)	7.67	$P2_12_12_1$	15.927	22.559	25.617	II	17.6
				Metal-Conjugates					
Co−CN−Re	2004	WAFJAP (35a)	6.62	$P2_12_12_1$	15.876	21.845	26.367	II	18.1
Co−CN−Re	2004	WAFJET (35a)	9.90	$P2_12_12_1$	15.958	21.233	27.978	none	19.0
Co−CN−Pt	2005	KARTUT (35b)	7.20	$P1$	16.943	17.312	18.081	N/A	4.6; 7.5
Co−CN−Pt	2007	NEYBAV (35c)	5.64	$P1$	16.922	17.298	18.101	N/A	5.7; 8.2
Co−CN−Pt	2005	KARVAB[b] (35b)	7.62	$P2_12_12_1$	14.094	24.935	27.278	none	18.4
Co−CN−Pt	2007	REYWIC (35d)	9.23	$P2_12_12_1$	15.963	21.538	26.633	II	18.7
Co−CN−Pt	2007	NEVSAJ (35c)	4.82	$C2$	33.832	17.282	16.995	N/A	7.1
Co−CN−Pt	2008	GODLAN (35e)	9.84	$P2_12_12_1$	15.706	22.412	25.571	II	18.9
Co−CN−Pt	2008	GODLER (35e)	8.92	$P2_12_12_1$	15.873	22.023	26.219	II	17.7

[a] CSD database search September 2010. [b] The a and c axis are inverted to resemble the conventional unit cell.

X group, and (ii) if crystal-packing forces can affect the molecular structure (axial bond lengths, corrin deformation). Although the answer to the first question is somewhat obvious, the matter has not been clearly described in the field, generating some confusion. On the other hand, answering the second question is challenging and is the crux of this investigation. Note that the difference between *crystal packing influence* and *random crystal packing effects* is critical to such an investigation and this will be particularly stressed throughout. In fact, the existence of a correlation between "crystal packing types" (vide infra) and molecular features, if established, would be an intriguing property of Cbl structures.

Crystal Packing Analysis. As well-known, the distribution of solvent and host molecules within Cbls crystals can vary significantly; however, Cbls tend to be isomorphous, that is, to crystallize in the same space group (orthorhombic $P2_12_12_1$) with a *standard* unit cell (a, b, and c edge values close to 15, 20, and 25 Å, respectively), showing a so-called "packing invariance".[12b] Interestingly, modified XCbl or even small molecule-conjugates still usually retain the same basic unit cell (see Table 4). Kratky et al. originally noted[12b] the existence of three recurrent "packing types" characterizing *standard* XCbl structures (with exceptions being MeCbl[15,16] and [H_2OCbl]-(ClO_4)[33]), corresponding to similar arrangements of Cbl molecules in the *ab* plane. The differences lie in the way the Cbl layers are stacked along the c axis, a consequence of slightly different corrin side chain orientations and intra/intermolecular hydrogen-bonds. More recently, a fourth packing type was suggested by Randaccio et al.[26]

Typically, by plotting the values of the cell ratios c/a versus b/a, Cbls exhibiting the same packing type appear to fall in the same "cluster" (group) of points.[12] A modification of the literature diagrams, including the structurally characterized XCbls reported to date (see Table 1, CSD database September 2010), is given in Figure 4a (structural data available only to the original researchers[12a,b,26] have been omitted).

Taking into account the values of their respective unit cell edges (see Table 4), **1** and **5** fall into cluster I, **2** and **3** into cluster II and **4** into cluster IV. This inconsistency is a clear sign that the nature of the X group cannot be used to discriminate between different packing typologies. Indeed, the two original CNCbl structures reported by Hodgkin et al. belong themselves to different clusters, that is, "air dried"[11d] CNCbl to cluster I and "wet"[11e] CNCbl to cluster II. This observation alone makes the tacit assumption that crystal packing is innocent in Cbl-crystallography somewhat surprising.

Two different views of the crystal packing of **1**−**5** are presented in Figures 5a and 6. Consistent with the fact that different clusters are representative of different packing organizations, similarities can be found between the crystal packing of **1** and **5** (cluster I), and **2** and **3** (cluster II) in either the *bc* (Figure 5) or the *ab* (Figure 6) crystallographic planes, as expected.

The crystal packing of **4** appears similar to that of **1** and **5** when viewed along the a axis (although differences in the shape of the cavity can be evidenced using a space-filling representation, see Figure 5b), but more similar to that of **2** and **3** when viewed along the c axis, with each Li^+

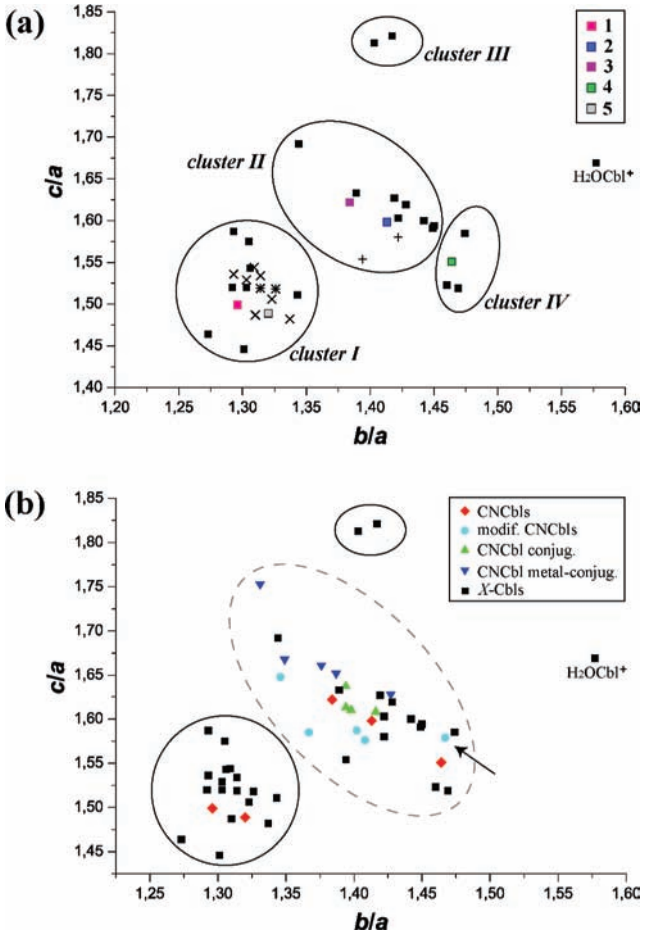

Figure 4. (a) Scatter plot of c/a versus b/a for the structurally characterized XCbl listed in Table 1. CNCbls **1**−**5** are evidenced with different colors. x = exceptions in cluster I (no intramolecular H-bond between the X group and the *c* amide side chain is observed); + = exceptions in cluster II (an intramolecular H-bond between the X group and the *c* amide side chain is observed). (b) Extended plot also incorporating modified CNCbls or CNCbls-conjugates with standard unit cell (see Table 4). The arrow indicates the position of norvitamin B_{12} (PEFWIH, ref 54), which seems to fall into cluster IV but does not show the two intramolecular H-bonds typical of this group.

cation connecting three neighboring molecules in **4** versus the larger K^+ only connecting two molecules in **5**. While the different connectivity shown by Li^+ or K^+ cations does not change the final dimensionality of the network of CNCbl molecules in **4** and **5** (3D in both cases), somehow only the presence of Li^+/Cl^- ions in **4**, but *not* of K^+/Cl^- in **5**, induces the formation of the intramolecular O8R-H···O51 H-bond.

It is interesting to note that our CNCbl-TFA adduct **1** and CNCbl·KCl (**5**) (as well as Hodgkin's "dry" B_{12}[11d]) both belong to cluster I, despite incorporating very different charged species. Conversely, both **2** and **3** crystallize according to cluster II, exhibiting the loss of the intramolecular N40−H···NC hydrogen bond observed in **1**, **4**, and **5**.

A series of incongruities has drawn our attention over the course of this analysis. For instance, it appeared well-established[12a] that XCbls belonging to cluster I are characterized by an intramolecular H-bond interaction between the X group and the amide functionality of the *c* side chain, analogously to XCbls of cluster IV. This led to the hypothesis that only XCbls with suitable X ligands (hydrogen bonds acceptors or donors) could be found in clusters I or IV. Conversely, alkylCbls were thought to belong only to clusters II or III.[12a] However, as recently discovered for Ethyl- (EtCbl) and Butyl- (BuCbl) cobalamin,[38] alkylCbls can also crystallize into cluster I, although with the loss of the "standard" H-bond. Moreover, the introduction of new points corresponding to the latest structural determinations[22,27,48] makes the distinction between clusters II and IV not as clear as previously reported. In Figure 4a, the two groups are shown still separated according to Randaccio et al., who first showed cluster IV being characterized by two distinctive intramolecular H-bonds involving (i) the amide N40 nitrogen atom (chain *c*) and the β-axial X group and (ii) the ribose O8R and the carbonyl O51 (chain *e*) oxygen atoms.[26] However, if a list of known modified CNCbls[49−54] or CNCbl-conjugates is included in the plot (Figure 4b), exceptions to this "rule" can be noted for cluster IV. In general, a discrete number of exceptions to general H-bonds motifs can be also found either in cluster I or II, as shown in Figure 4a.

These observations strongly suggest an important level of unpredictability in the solid-state chemistry of cobalamins, with random crystal packing effects most likely playing an important role.

Molecular Analysis of 1−5 and Correlation with Crystal Packing. A superimposition of the five CNCbl molecules in the crystal structures under investigation is proposed in Figure 7.[55]

Analysis of the cobalt coordination environment in **1**−**5** (see Table 3) reveals a certain consistency among the equatorial Co−N distances, while a larger variation affects the two axial bonds. A similar trend can be noticed in the cobalt equatorial plane for **1**−**4**: Co1−N21 ≤ Co1−N24 < Co1−N22 ≤ Co1−N23, with values typically oscillating around 1.88, 1.90, 1.91, and 1.92 Å, respectively. In each of these cases, the Co−N distances within rings A (N21) and D (N24) are shorter by about 0.02 Å with respect to ring B (N22) and D (N23), as normally observed.[12a] A different type of equatorial distortion is exhibited,

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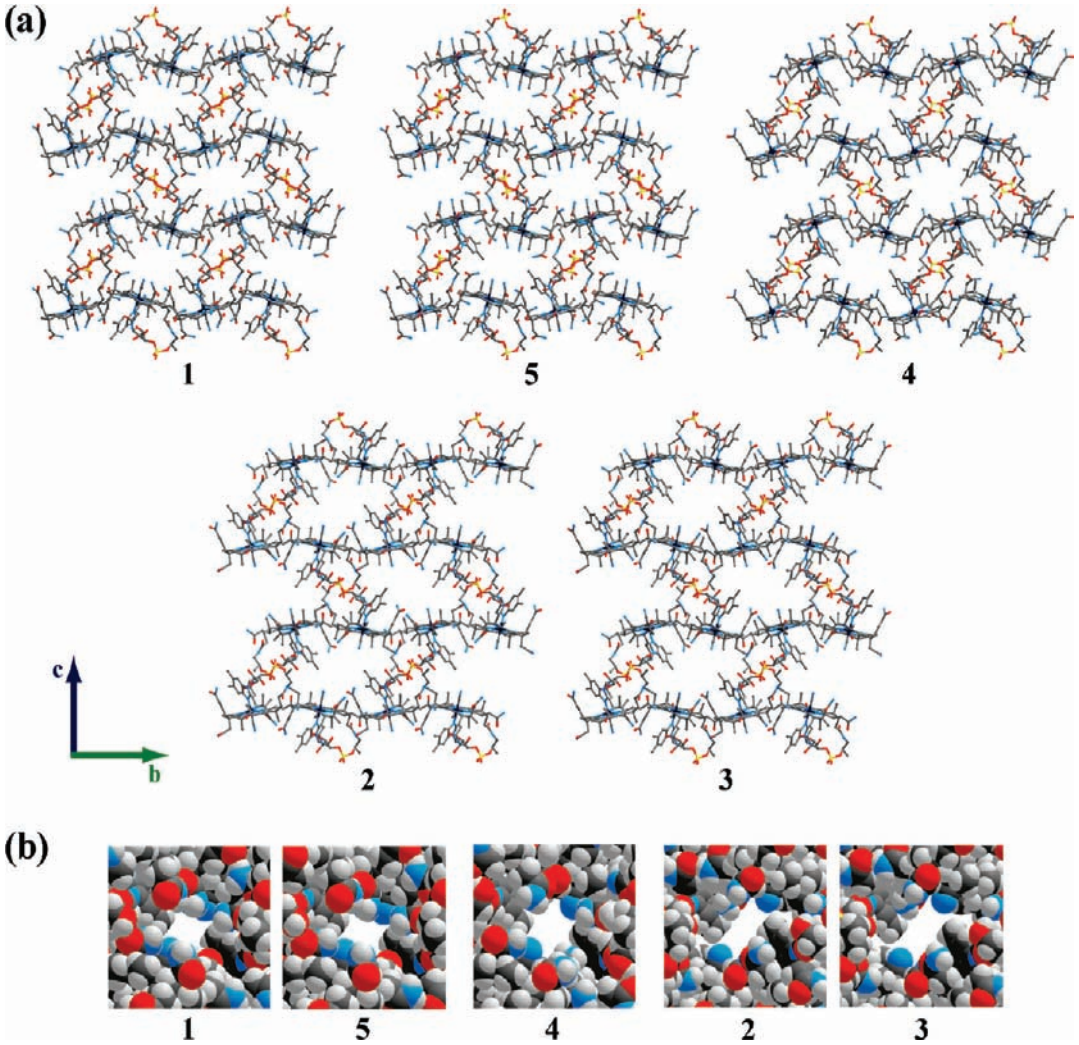

Figure 5. (a) Crystal packing of **1**−**5** viewed along the crystallographic *a* axis. Hydrogen atoms, solvent and ionic content not shown. (b) Space filling view of the channels running along *a* in **1**−**5**. Note the similarities between **1**/**5**/**4** and **2**/**3**.

instead, by **5**, with a very short Co1−N21 bond (ca. 1.86 Å) and three similar values for the Co1−N24, Co1−N22, and Co1−N23 distances (ca. 1.90 Å). The two axial bonds approximately oscillate in the ranges 1.86−1.89 Å (Co−C64, Δ = 0.03 Å) and 2.01−2.05 Å (Co−N3B, Δ = 0.04 Å), with major correlation between CNCbls **1**/**5**, **2**/**4** and **3**/**5**.

Interestingly, as **1** and **5** show the same solid-state arrangement (cluster I), they also show no practical differences in the length of the axial fragments, suggesting the possibility to correlate crystal packing with molecular structure; unfortunately, the existence of a similar correlation is questionable in the case of **2** and **3** (cluster II), the latter structure being much less accurate than the former (compare e.s.d.'s values in Table 3).

In general, if the reported experimental error are taken in consideration, the set becomes quite uniform and little difference can be observed in the CNCbl cobalt(III) environment within the five structure. However, the observed folding in **1**−**5** ranges from 14.1° in **5** to 18.7° in **4**, a variation of about 5°, which cannot, we believe, be attributed solely to crystallographic inaccuracy (see also, for instance, the discussion about the folding in ref 12a). The upward folding (ϕ) of the corrin macrocycle is an important structural parameter for Cbls, and is defined[12a,c] as the dihedral angle between the planes passing through N21, C4, C5, C6, N22, C9, C10 and C10, C11, N23, C14, C15, C16, N24 (Figure 7a). Given that the similarities noted in the cobalt(III) coordination sphere in **1**−**5** surely lead to some ambiguities in the attempt to correlate crystal packing type and axial fragment properties, it seems interesting to explore whether or not at least a correlation can be found between packing types and corrin folding. Crystal packing analysis showed that **1** and **5** belong to the same cluster, as well as **2** and **3**. As indicated in Table 4, the corrin folding angle is 15.55(5)° and 14.1° in **1** and **5**, respectively, while it is 15.9° and 18.0° in **2** and **3** [e.s.d.'s values not available for **2**−**5**]. Once again, the numbers suggest there are no significant correlations between crystal packing motifs and molecular properties. When comparing CNCbls with similar folding properties, such as **1**/**2**/**5** (ϕ in the range 14−16°) and **3**/**4** (ϕ in the range 18−19°), this becomes even more evident. In fact, as shown in Figure 7 (b-c), (i) the orientation of chains *a*, *c*, *d*, and *e* is very different in **2** with respect to **1**/**5**, and also (ii) the orientation of chains *b*, *c*, *e*, and the ribosyl C5R-O8R pendant is different in **3** or **4**, this being an indication of the variability of crystal packing within the considered series. By participating in hydrogen-bonding interactions

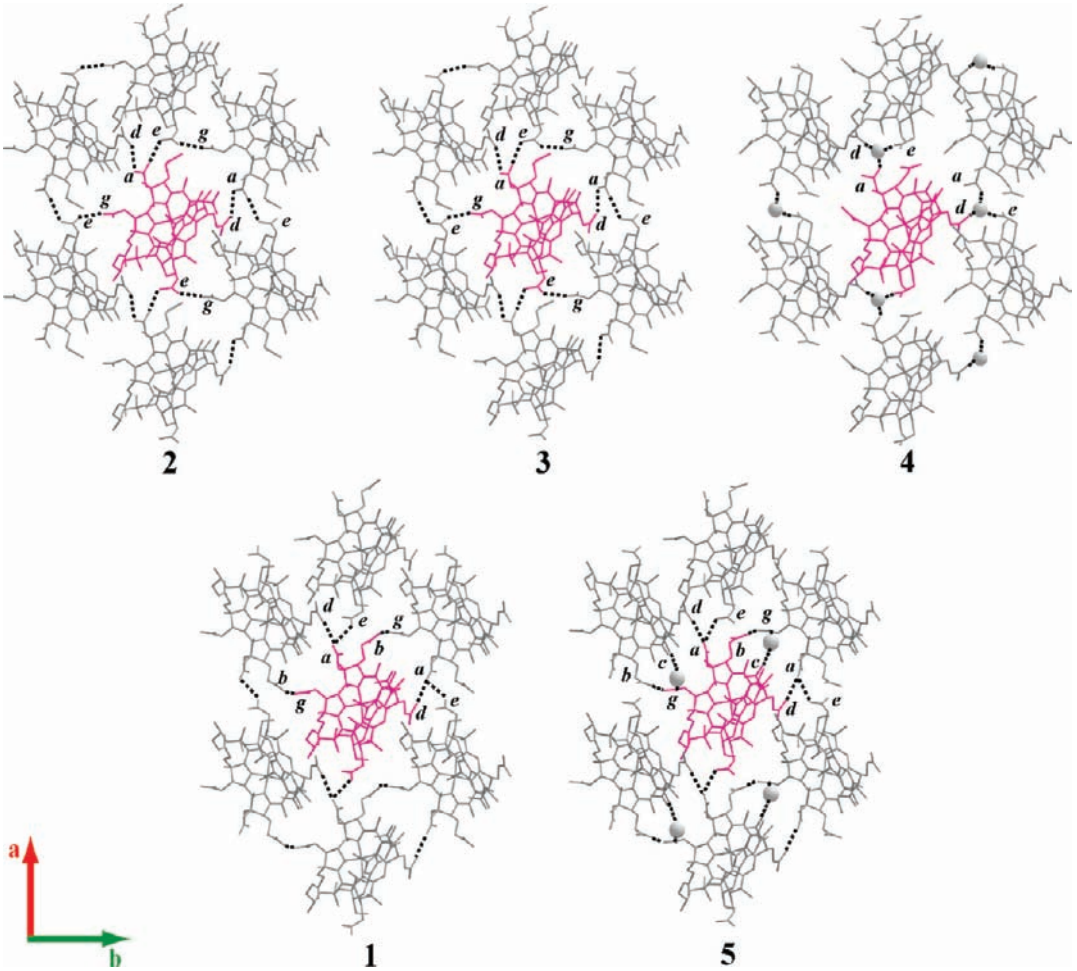

Figure 6. Crystal packing of **1**−**5** viewed along the crystallographic *c* axis (single layer). Lithium and potassium cation in **4** and **5** are represented as gray spheres. Solvent molecules and anionic content are omitted for clarity. In **2** and **3**, direct contact between the side chains of neighboring molecules are observed as *a*-*d*, *a*-*e*, and *e*-*g*. In **4**, no direct contacts are observed, but each Li^+ cation links together three adjacent Cbl molecules by coordination through side chains *a*, *d*, and *e*. In **1** and **5**, direct contact between the side chains of neighboring molecules are observed as *a*-*d*, *a*-*e*, and *b*-*g*. An extra, potassium-mediated, *b*-*c* contact is observed in **5**. Note the similarities in the *ab* dimensions for **1**/**5** and **2**/**3**/**4** (see also Table 4).

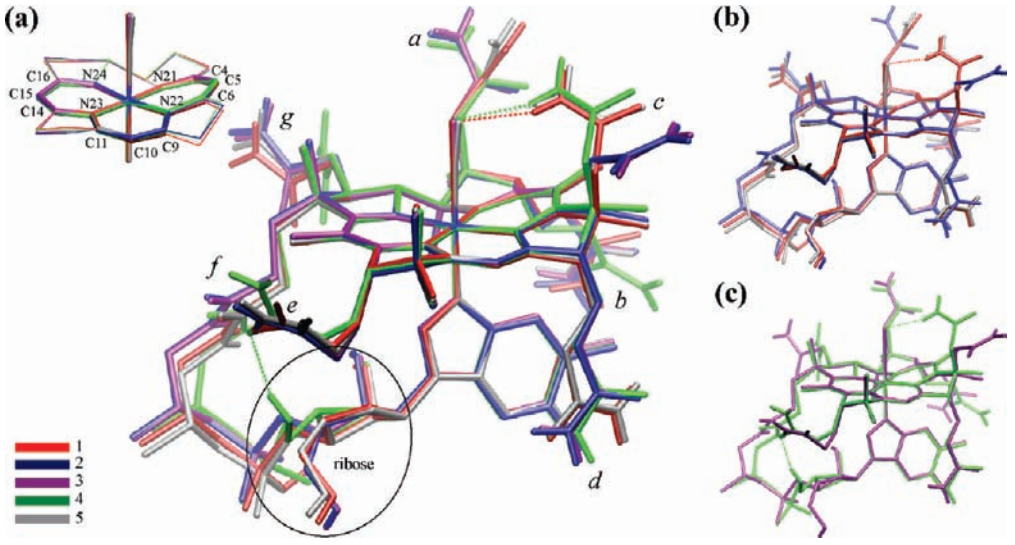

Figure 7. (a) Superimposition of the CNCbl molecules in **1**−**5** (alignment performed using the cobalt ion and the atoms in its octahedral environment). A closer look at the corrin, highlighting the two sets of atoms that form the two planes involved in the calculation of the fold angle ϕ, is given as inset. (b) Superimposition of selected structures with ϕ angle in the range 14−16° (cobalamins **1**, **2**, and **5**). (c) Superimposition of selected structures with ϕ angle in the range 18−19° (cobalamins **3** and **4**).

then, the corrin side chains can significantly affect corrin deformation (see Supporting Information, Figure S2 and S3).

Conclusions

An unprecedented CNCbl-trifluoracetate salt (**1**) has been isolated in the solid state and its crystal structure has been analyzed through standard X-ray techniques (MoK_α radiation), obtaining a very low crystallographic residual ($R = 4.1\%$). The cobalamin is in the "base-on" conformation, being the TFA proton transferred to the phosphate group. A fully ordered pattern of water molecules of crystallization and anions is found in **1**, something rarely observed in cobalamin structures.[12] Given the ease with which crystals were obtained and the additional stability provided to the crystal packing by the multiple hydrogen bonds involving the $TFAc^-$ anion, we believe this TFA "spiking" method may represent a facile route to high quality Cbl crystals.

To investigate the influence of the protonation on the CNCbl molecule, we compared **1** with four other previously reported CNCbl structures of high crystallographic quality (**2**−**5**).[13−15] We found that the hydrogen-bonding network in the proximity of the phosphate group is not significantly influenced by the presence/absence of a protonated phosphate-oxygen atom; therefore, **1** can be considered a CNCbl iono-solvate, akin to **2**−**5**.

The analysis of the crystal packing of **1**−**5** reveals the five structures belong to different "packing types".[12] Unexpectedly, despite profound differences in the respective iono-solvate content, the crystal packing of **1** and **5** match very well, showing that casual effects are critical in the solid state chemistry of Cbls.

At a molecular level, we detected only slight variations in the cobalt(III) coordination environment within **1**−**5**, but pronounced differences in the "upward" corrin folding, which cannot be justified on the basis of axial bond lengths. A closer look at the CNCbl molecule in its entirety shows that, by participating in crucial hydrogen-bonding interactions, corrin side chains can also significantly affect the corrin deformation. In the end, the observed analogies/differences among **1**−**5** should be attributed to random packing effects. No significant correlation between crystal packing motifs and molecular properties was observed.

Our findings support and complement the recent computational reports, involving AdoCbl, that suggests the existence of a relationship between crystal packing forces and Cbl molecular structure.[41a] In fact, we demonstrate here that crystal packing forces are influential even in the presence of a much less bulky β-group as CN^-. As far as we know, this is the first time that an in-depth structural investigation has been performed on a series of different iono-solvates of the same cobalamin species. Indeed, a great level of unpredictability has been found in the solid state chemistry of cobalamins that challenges our basic understanding of such structures and suggests a need to reexamine previous assumptions, especially if considering the use of the solid state approach to study the biologically relevant properties of these systems.

Certainly we believe this topic deserves further investigation both from a structural and a computational perspective if we are to truly understand this unique molecule.

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Supporting Information Available: Crystallographic data in CIF format; Table S1; hydrogen bonds details (Figure S1, Table S2); Figures S2−S3. This material is available free of charge via the Internet at http://pubs.acs.org.