Phytochrome as molecular machine: Revealing chromophore action during the Pfr→Pr photoconversion by magic-angle spinning NMR spectroscopy

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Fig. S1: Contour plot of the 2D ^{13}C - ^{13}C DARR NMR spectra of u- ^{13}C , ^{15}N -PCB-Cph1 Δ 2 in the Lumi-F intermediate state. The spectra were recorded with mixing times of 5 ms (blue) and 50 ms (green) at 173K and a spinning frequency of 9000 and 8500 Hz, respectively.

Fig. S2: Contour plot of the 2D ^{13}C - ^{13}C DARR NMR spectra of u- ^{13}C , ^{15}N -PCB-Cph1 Δ 2 in the Meta-F intermediate state. The spectra were recorded with mixing times of 5 ms (red) and 50 ms (purple) at 203K and a spinning frequency of 9000 and 8500 Hz, respectively.

Fig. S3: Structural formula of protein bound PCB shown in the *ZZZssa* (A) and *ZZEssa* (B) geometries, as assumed by the Pr (pdb 2VEA) and Pfr (pdb 3C2W) states, respectively.

Fig. S4: Contour plot of the ^1H - ^{15}N HMBC NMR dipolar correlation spectrum of phycocyanobilin in d_4 -methanol recorded in a magnetic field of 14.1 T at 277 K.

Atom	Pfr	Lumi-F	Meta-F	Pr
1a	182.8	183.2	183.6	182.5
1b	—	—	—	184.0
2	37.2	37.3	37.1	37.1
2a	—	—	—	37.9
2 ¹	18.5	18.6	18.6	17.5
3	54.3	54.3	54.0	53.4
3 ¹	50.0	50.0	49.2	47.6
3 ²	21.4	21.9	22.0	21.8
4	154.0	n.d.	154.2	153.9
5	88.5	88.5	87.5	87.1
6	148.8	n.d.	n.d.	149.1
7	126.1	126.7	126.1	126.0
7 ¹	9.3	9.5	9.3	9.2
8	143.6	144.6	145.5	144.5
8 ¹ a	23.1	23.4	22.0	22.5
8 ¹ b	—	—	—	21.5
8 ² a	41.7	42.6	42.8	43.1
8 ² b	—	—	—	41.1
8 ³ a	180.5	180.8	180.6	180.5
8 ³ b	—	—	—	179.7
9	131.0	130.1	128.1	127.7
10	112.3	113.8	112.9	112.7
11	131.0	130.1	128.1	127.7
12	145.8	145.7	145.5	145.5
12 ¹	20.6	21.0	21.0	21.1
12 ²	38.3	40.0	38.3	37.5
12 ³	175.2	178.2	179.1	179.5
13	130.6	129.9	126.2	126.3
13 ¹	11.3	11.5	11.5	11.3
14	151.5	149.5	145.4	145.7
15	91.7	91.9	93.2	93.5
16	151.1	149.5	145.4	145.7
17	137.7	139.8	143.2	142.2
17 ¹	10.1	10.1	10.2	9.8
18	140.4	134.4	134.2	134.3
18 ¹	15.6	15.6	16.1	16.1
18 ²	13.3	n.d.	n.d.	13.3
19	168.9	171.8	172.7	172.8

n.d.: not determined

Table S1: The ¹³C chemical shift assignments obtained in the present work.

Atom	Pfr	Lumi-F	Meta-F	Pr	PCB
N21	158.6	157.9	156.0	158.1	157.5
N22	155.8	157.9	161.1	160.5	245.3
N23	142.6	146.0	144.8	147.0	152.7
N24	138.0	136.5	127.1	131.9	130.9

Table S2: Tentative ^{15}N chemical shift assignments obtained in the present work.

Tentative ^{15}N assignment

Nitrogen atom assignments on $^{15}\text{N21-PCB-Cph1}\Delta 2$

The one-dimensional (1D) ^{15}N spectra of $^{15}\text{N21-PCB-Cph1}\Delta 2$ labeled phytochrome in the Pfr, Lumi-F, Meta-F and Pr states reveal that $^{15}\text{N21}$ resonates at around 158. The evidence obtained with a selectively $^{15}\text{N21-PCB}$ labeled sample now allows more precise ^{15}N assignments than those that were formerly proposed (1, 2). Previous assignment was based on the assumption that the hydrogen-bonding is symmetric and the positive charge is equally distributed over both nitrogens of rings **B** and **C** (N22 and N23, respectively), leading to very similar chemical shifts between 155 and 160 ppm. The assignment of the signal at 158 ppm to N21, however, makes such an assignment impossible. Therefore we conclude that the two nitrogen atoms of the inner rings appear at different chemical shift values, probably due to either different hydrogen-bonding interactions or a more distinctly located positive charge on the ring **B** or the ring **C** nitrogen.

Nitrogen atom assignments on model compounds

Further insights into the ^{15}N chemical shift assignment are obtained by liquid-state NMR. The ^1H - ^{15}N heteronuclear multiple bond correlation (HMBC) spectrum of u- $(^{13}\text{C}, ^{15}\text{N})$ -PCB in its free state is shown in Fig. S4. The ^1H chemical shifts of the methine bridges (3) allow the ^{15}N resonances to be assigned (Table S2). In solution, the ring **D** nitrogen (N24) resonance occurs at 130.9 ppm, supporting our previous assignment of the ^{15}N signals at 131.9 and 138.0 ppm in the Pr and Pfr states to N24. In addition, the ^{15}N resonances at 136.5 and 127.1 ppm are assigned to N24 in the Lumi-F and Meta-F

intermediates states, respectively. The changes in ^{15}N chemical shifts of N24 are confirmed by DFT calculations (see below). The two remaining ^{15}N signals originate from N22 and N23. The X-ray structure of Cph1 shows that the amide oxygen of Asp-207 is at hydrogen-bonding distance to N21, N22 and N23. Especially, the distance between this oxygen and N22 is short as 2.72 Å, *i.e.*, shorter by three times than the standard deviation of the average $\text{N}\cdots\text{O}$ distance in a $\text{N}-\text{H}\cdots\text{O}$ hydrogen-bonding interaction (4). It is known that the hydrogen-bonding interaction between a carbonyl group and a cationic nitrogen ($\text{C}=\text{O}\cdots\text{H}-\text{N}(+)$) is stronger than in the case of a non-charged nitrogen ($\text{C}=\text{O}\cdots\text{H}-\text{N}$) (5). In addition, the bonding energy of a hydrogen-bonding interaction is inversely related to the distance between the involved atoms. This asymmetry of the hydrogen-bonding environment may explain that the ^{15}N chemical shift of N22 is low-field shifted relative to the one of N23. Therefore, we tentatively assign the peaks at 160.5 and 147.0 to N22 and N23, respectively. In analogy, the signal at 155.8 and 142.6 are respectively assigned to N22 and N23 in the Pfr state.

DFT calculation on small model compounds of ring *D*

As a simple test for hydrogen-bonding effects on the changes in chemical shifts at ring *D* in phytochrome, isotropic chemical shieldings have been calculated for models 1 to 4 in Fig. S5. Model 1 which represents the Pfr state contains a hydrogen bond between a propionic acid molecule and the N–H and between the carbonyl group and a tyrosine. In model 2, there is a hydrogen bond between a propionic acid molecule and the N–H. Model 3 corresponds to the isolated ring *D*, as assumed for Meta-F. Model 2 which presents a hydrogen bond from the N–H group to a water molecule (the N–H $\cdots$ O angle was constrained in that optimization) stands for the Pr state.

Fig. S5: Optimized structures (BP86/TZP) of additional small model compounds for the phycocyanobilin chromophore in phytochrome. Note that Cs symmetry was imposed in models 1, 2 and 4.

All structures are optimized with the Becke-Perdew exchange-correlation functional (6, 7) denoted as BP86 using the NMR module (8, 9, 10) of the Amsterdam Density Functional Program (ADF) (11, 12). The TZP basis set from the ADF basis set library

was employed throughout. Tests with the much larger QZ4P basis set showed only minor differences in the relative chemical shifts. Isotropic chemical shifts and changes in chemical shieldings have been calculated with BP86/TZP and SAOP/TZP. The more sophisticated SAOP (“statistical averaging of model (orbital) potentials”) potential (13, 14, 15) is expected to yield more reliable chemical shift information (Table S3). The $\Delta\delta$ values indicate that in particular for N1, C2, and C6 the chemical shift decreases ($\equiv$ the shielding increases) when the hydrogen bond is removed.

atom	σ (1)	σ (2)	σ (3)	σ (4)	$\Delta\delta$ (1-2)	$\Delta\delta$ (2-3)	$\Delta\delta$ (3-4)
BP86/TZP							
N1	91.11	92.11	99.36	92.05	1.00	7.25	-7.31
C2	7.67	9.69	11.86	9.66	2.02	2.17	-2.20
C3	53.73	53.84	53.91	54.02	0.11	0.07	0.11
C4	49.61	50.08	48.96	49.34	0.47	-1.12	0.38
C5	34.95	34.57	35.84	35.25	-0.38	1.27	-0.59
C6	64.33	70.63	71.76	71.44	6.30	1.13	-0.32
C7	167.43	168.00	168.15	168.04	0.57	0.15	-0.11
SAOP/TZP							
N1	96.08	98.12	103.96	98.26	2.04	5.84	-5.70
C2	12.23	13.46	15.37	13.30	1.23	1.91	-2.07
C3	57.58	57.00	57.04	57.16	-0.58	0.04	0.12
C4	51.66	51.85	50.78	51.10	0.19	-1.07	0.32
C5	37.89	36.96	38.29	37.58	-0.93	1.33	-0.71
C6	65.60	70.46	71.59	71.25	4.86	1.13	-0.34
C7	168.07	168.54	168.73	168.58	0.47	0.19	-0.15

Table S3: Isotropic shieldings σ for the carbon and nitrogen atoms in the model compounds 1 to 4 as well as differences in chemical shifts $\Delta\delta = -\Delta\sigma$ between the different compounds.

The comparison between the calculated and experimental ^{15}N chemical shift of N24 is shown in Table S4. The variation of the ^{15}N chemical shift obtained by MAS NMR during the three transitions follow the same trends than the calculated difference in chemical shifts.

$\Delta\delta$ (1-2)	$\Delta\delta$ (2-3)	$\Delta\delta$ (3-4)	$\Delta\delta 1$	$\Delta\delta 2$	$\Delta\delta 3$
	BP86/TZP			Experimental	
1.00	7.25	-7.31	1.5	9.4	4.8
	SAOP /TZP			Experimental	
2.04	5.84	-5.70	1.5	9.4	4.8

Table S4: Comparison of the calculated and experimental differences in ^{15}N chemical shifts $\Delta\delta$ of N24 between the different compounds/states. $\Delta\delta 1 = \Delta\delta(\text{Pfr-LumiF})$, $\Delta\delta 2 = \Delta\delta(\text{LumiF-MetaF})$ and $\Delta\delta 3 = \Delta\delta(\text{MetaF-Pr})$.

Synthesis of $^{15}\text{N21-PCB-Cph1}\Delta 2$

Chemical Section

General Methods: Column chromatography: Purifications were performed on Merck silica gel 60 (230–400 mesh) under slightly increased pressure. HPLC: Purifications were carried out on following columns: Column A [Macherey-Nagel Nucleosil 5 μ C18 (RP-C₁₈, 5 μ m, 250 mm $\times$ 8 mm, flow rate 0.8 ml min⁻¹)], column B [Phenomenex Luna 5 μ C18 (RP-C₁₈, 5 μ m, 150 mm $\times$ 4.6 mm, flow rate 1.2 ml min⁻¹)], column C [Phenomenex Gemini 5 μ C18 (RP-C₁₈, 5 μ m, 150 mm $\times$ 4.6 mm, flow rate 1.2 ml min⁻¹)]. NMR: Spectra were recorded on Bruker ARX 250, DRX 400, or DRX 500 spectrometers in CDCl₃; chemical shifts (δ) are given in ppm relative to TMS, coupling constants (J) in Hz. The solvent signals were used as references and the chemical shifts converted to the TMS scale (CDCl₃: δ_{C} = 77.0 ppm; residual CHCl₃ in CDCl₃: δ_{H} = 7.24 ppm). IR: Perkin-Elmer System 2000 spectrometer; wavenumbers $\tilde{\nu}$ in cm⁻¹. UV/Vis: Shimadzu UV-2401 PC spectrometers; the solvents used were of appropriate quality (Uvasol). MS (EI): Finnigan MAT 8200, or MAT 8400 (70 eV); ESI-MS: Bruker Esquire 3000, or HP Quadrupol MS Engine; accurate mass determinations (HRMS): Finnigan MAT 95. Melting points: Reichert melting point microscope.

Synthesis scheme

2-(4-Methoxybenzyl)(^{15}N)-1*H*-isoindole-1,3(2*H*)-dione (1**):** A dispersion of ^{15}N -labeled potassium phthalimide (10.00 g, 53.7 mmol), 1-(Chloromethyl)-4-methoxybenzene (6.1 ml, 44.8 mmol), and 18-crown-6 (1.18 g, 4.5 mmol) in toluene (58 ml) was stirred under argon at 100 °C for 5 h. After cooling to ambient temperature, water (150 ml) was added to the mixture. The resulting phases were separated, the aqueous phase was extracted with dichloromethane (4 × 50 ml) and the combined organic phases were dried over Na_2SO_4 . Evaporation of the solvent and drying of the remaining pale yellow solid under reduced pressure yielded the reaction product **1** (12.01 g, 44.8 mmol, 100 %) which could be used in the following reaction without further purification. m.p. 132 °C; ^1H -NMR (400 MHz, CDCl_3): δ [ppm] = 3.75 (s, 3 H, 1- CH_3), 4.76 (s, 2 H, 6- CH_2), 6.82 (d, $^3J_{\text{H,H}} = 8.62$ Hz, 2 H, 2 × 3- CH), 7.36 (d, $^3J_{\text{H,H}} = 8.63$ Hz, 2 H, 2 × 4- CH), 7.67 (dd, $J_{\text{d}} = 3.03$ Hz, $^3J_{\text{H,H}} = 5.46$ Hz, 2 H, 2 × 10- CH), 7.81 (dd, $J_{\text{d}} = 3.09$ Hz, $^3J_{\text{H,H}} = 5.37$ Hz, 2 H, 2 × 9- CH); ^{13}C -NMR (101 MHz, $^1\text{H}/^{13}\text{C}$ -COSY, CDCl_3): δ [ppm] = 41.05 (d, $J = 9.27$ Hz, C-6), 55.25 (C-1), 114.01 (C-3), 123.28 (C-9), 128.68 (C-5), 130.14 (C-4),

132.20 (d, $J = 7.71$ Hz, C-8), 133.91 (C-10), 159.24 (C-2), 168.06 (d, $J = 12.93$ Hz, C-7); IR (KBr): $\tilde{\nu} = 3464, 3002, 2969, 2935, 2840, 1768, 1719, 1612, 1513, 1466, 1429, 1386, 1355, 1334, 1319, 1305, 1289, 1249, 1176, 1099, 1087, 1033, 958, 822, 808, 823, 749, 723, 713, 640, 619, 566, 529, 508\text{ cm}^{-1}$; MS (EI, 90 °C): m/z (%): 268 (100) $[M]^+$, 250 (7), 237 (7), 225 (21), 211 (5), 181 (5), 161 (2), 135 (20), 121 (10), 108 (12), 91 (4), 77 (13), 65 (2), 51 (3), 39 (1); HRMS (ESI pos, $\text{CH}_2\text{Cl}_2 + \text{CH}_3\text{OH}$): m/z : calcd for $\text{C}_{16}\text{H}_{13}^{15}\text{NNaO}_3 [M + \text{Na}]^+$: 291.075794; found: 291.075878.

1-(4-Methoxyphenyl)methan(^{15}N)amine (2): A dispersion of **1** (6.41 g, 23.9 mmol) and hydrazine hydrate (51 % hydrazine, 96 ml, 1.0 mol) in methanol (1.4 l) was refluxed for 5 h. After evaporation of the solvent under reduced pressure the remaining oil was dissolved in dichloromethane (500 ml), washed with NaOH (1 M, 5×100 ml) and dried over Na_2SO_4 . Evaporation of the solvent under reduced pressure yielded the reaction product **2** (3.20 g, 23.2 mmol, 97 %) as a yellow, clear oil. ^1H -NMR (400 MHz, CD_3OD): δ [ppm] = 3.69 (s, 2 H, 1- CH_2), 3.75 (s, 3 H, 6- CH_3), 6.86 (d, $^3J_{\text{H,H}} = 8.70$ Hz, 2 H, 3- CH), 7.22 (d, $^3J_{\text{H,H}} = 8.71$ Hz, 2 H, 4- CH); ^{13}C -NMR (101 MHz, $^1\text{H}/^{13}\text{C}$ -COSY, CD_3OD): δ [ppm] = 44.69 (C-1), 54.31 (C-6), 113.55 (C-4), 128.32 (C-3), 134.21 (C-2), 158.82 (C-5); IR (KBr): $\tilde{\nu} = 3277, 2938, 2840, 2637, 2182, 1613, 1598, 1583, 1555, 1516, 1469, 1457, 1439, 1400, 1380, 1327, 1292, 1252, 1184, 1108, 1025, 985, 834, 809, 749, 686, 644, 612, 559, 542, 508\text{ cm}^{-1}$; MS (EI, -20 °C): m/z (%): 138 (65) $[M]^+$, 137 (100), 121 (31), 107 (35), 94 (21), 91 (7), 77 (17), 65 (7), 51 (7), 39 (6), 31 (13); HRMS (CI, *i*-Butan): m/z : calcd for $\text{C}_8\text{H}_{12}^{15}\text{NO} [M + \text{H}]^+$: 139.088923; found: 139.088793.

In general, the preparation of **6** follows the synthetic route of the unlabeled ring A compound established by Kakiuchi *et al.* (16).

1-(4-Methoxybenzyl)-3-methyl(^{15}N)-1H-pyrrole-2,5-dione (3): A solution of **2** (5.89 g, 42.6 mmol) in toluene (15 ml) was added slowly to a cooled solution of citraconic anhydride (3.9 ml, 42.5 mmol) in toluene (250 ml) under argon. The mixture was refluxed for 17 h and afterwards the solvent was evaporated under reduced pressure. The resulting oil was dissolved in ethyl acetate (50 ml) and washed with water (5×50 ml)

and brine (2 × 50 ml). After drying over Na₂SO₄, evaporation of the solvent yielded the crude product which was purified by column chromatography with *n*-hexane and ethyl acetate (gradient 1:0–3:1, v/v) as eluent yielded the reaction product **3** (6.75 g, 29.1 mmol, 68 %) as pale yellow solid. m.p. 72–73 °C; ¹H-NMR (400 MHz, CDCl₃): δ [ppm] = 2.04 (d, ⁴J_{H,H} = 1.84 Hz, 3 H, 3¹-CH₃), 3.75 (s, 3 H, 1⁶-CH₃), 4.56 (s, 2 H, 1¹-CH₂), 6.28 (q, ⁴J_{H,H} = 1.51 Hz, 1 H, 4-CH), 6.81 (d, ³J_{H,H} = 8.69 Hz, 2 H, 1⁴-CH), 7.27 (d, ³J_{H,H} = 8.64 Hz, 2 H, 1³-CH); ¹³C-NMR (101 MHz, ¹H/¹³C-COSY, CDCl₃): δ [ppm] = 10.92 (C-3¹), 40.92 (d, J_{C,H} = 9.71 Hz, C-1¹), 55.23 (C-1⁶), 113.96 (C-1⁴), 127.34 (d, J_{C,H} = 6.96 Hz, C-4), 128.74 (C-1⁵), 129.89 (C-1³), 145.71 (C-3), 159.17 (C-1⁵), 170.43 (C-5), 171.44 (C-2); IR (KBr): $\tilde{\nu}$ = 3453, 3085, 3007, 2974, 2943, 2843, 1708, 1696, 1638, 1613, 1586, 1510, 1463, 1440, 1384, 1345, 1306, 1286, 1246, 1181, 1102, 1067, 1030, 945, 875, 854, 820, 771, 760, 732, 701, 647, 635, 603, 578, 535 cm⁻¹; MS (EI, 55 °C): *m/z* (%): 232 (100) [M]⁺, 214 (2), 204 (6), 189 (37), 175 (4), 161 (6), 135 (17), 121 (18), 108 (38), 91 (6), 77 (10), 68 (4), 51 (4), 39 (9), 29 (1); HRMS (ESI pos, CH₂Cl₂ + CH₃OH): *m/z*: calcd for C₁₃H₁₃¹⁵NNaO₃ [M + Na]⁺: 255.075801; found: 255.07900.

(3E)-3-Ethylidene-1-(4-methoxybenzyl)-4-methyl(¹⁵N)pyrrolidine-2,5-dione (4): To a solution of DBU (4.4 ml, 29.1 mmol) in nitroethane (69 ml, 0.94 mol) which was cooled at 0 °C **3** (6.75 g, 29.1 mmol) was added in one portion. The resulting mixture was stirred for 1 h at 0 °C. Addition of ethyl acetate (130 ml) was followed by washing with water (4 × 130 ml) and brine (2 × 130 ml). After drying over Na₂SO₄, evaporation of the solvent yielded the crude product (8.69 g). This was purified by column chromatography repeatedly with *n*-hexane and ethyl acetate (gradient 9:1–3:1, v/v) as eluent yielding the reaction product **4** (3.33 g, 12.8 mmol, 44 %) as pale yellow oil and its (3*Z*)-isomer (0.69 g, 2.7 mmol, 9 %) as pale yellow solid. ¹H-NMR (400 MHz, CDCl₃, NOESY): δ [ppm] = 1.36 (d, ³J_{H,H} = 7.46 Hz, 3 H, 4¹-CH₃), 1.86 (dd, ³J_{d,H,H} = 7.33 Hz, J_d = 1.22 Hz, 3 H, 3²-CH₃), 3.26 (q, ³J_{H,H} = 7.38 Hz, 1 H, 4-CH), 3.74 (s, 3 H, 1⁶-CH₃), 4.61 (dd, J_d = 3.54 Hz, J_d = 0.75 Hz, 2 H, 1¹-CH₂), 6.79 (d, ³J_{H,H} = 8.73 Hz, 2 H, 1⁴-CH), 6.82 (dq, J_d = 2.16 Hz, ³J_{q,H,H} = 7.33 Hz, 1 H, 3¹-CH), 7.30 (d, ³J_{H,H} = 8.67 Hz, 2 H, 1³-CH); ¹³C-NMR (101 MHz, ¹H/¹³C-COSY, CDCl₃): δ [ppm] = 14.65 (C-3²), 15.55 (C-4¹), 37.29 (d, J_{C,H} =

5.56 Hz, C-4), 41.54 (d, $J_{C,H}$ = 8.78 Hz, C-1¹), 55.16 (C-1⁶), 113.89 (C-1⁴), 128.31 (C-1²), 130.09 (C-1³), 132.00 (d, $J_{C,H}$ = 7.94 Hz, C-3), 134.05 (C-3¹), 159.16 (C-1⁵), 169.09 (C-2), 177.77 (d, $J_{C,H}$ = 10.90 Hz, C-5); IR (KBr): $\tilde{\nu}$ = 3457, 2977, 2939, 2838, 1766, 1706, 1676, 1614, 1586, 1551, 1515, 1457, 1431, 1385, 1341, 1293, 1249, 1198, 1178, 1142, 1112, 1074, 1033, 982, 949, 917, 849, 820, 768, 739, 699, 617, 570, 546, 515 cm⁻¹; MS (EI, 60 °C): m/z (%): 260 (100) [M]⁺, 245 (1), 231 (6), 217 (27), 203 (10), 187 (4), 163 (3), 152 (2), 135 (14), 121 (99), 108 (5), 96 (16), 77 (9), 68 (21), 53 (7), 41 (9); HRMS (EI): m/z : calcd for C₁₅H₁₇¹⁵NO₃ [M]⁺: 260.117880; found: 260.117585.

(3E)-3-Ethylidene-4-methyl(¹⁵N)pyrrolidine-2,5-dione (5): To a solution of **4** (3.33 g, 12.8 mmol) in acetonitrile (129 ml) and water (43 ml) CAN (17.63 g, 32.0 mmol) was added. After 3 h stirring at ambient temperature the solution was diluted with ethyl acetate (150 ml) and the resulting phases were separated. The aqueous phase was extracted with ethyl acetate (6 × 25 ml), and the combined organic phases were washed with a saturated solution of NaHCO₃ (4 × 100 ml) and brine (4 × 100 ml). After drying over Na₂SO₄, evaporation of the solvent yielded a yellow-brown solution which was dissolved in methanol (38 ml). Hydrazine hydrate (51 % hydrazine, 0.5 ml, 5.2 mmol) was added, the solution was stirred for 5 min at ambient temperature, and the solvent was evaporated under reduced pressure afterwards. The resulting orange yellow solid was separated between ethyl acetate (150 ml) and water (150 ml), the aqueous phase was extracted with ethyl acetate (3 × 50 ml), and the combined organic phases were washed with brine (4 × 100 ml). After drying over Na₂SO₄, evaporation of the solvent yielded the crude product (2.90 g). This was purified by column chromatography repeatedly with dichloromethane and ethyl acetate (gradient 19:1–4:1, v/v) as eluent yielding the reaction product **5** (0.24 g, 1.7 mmol, 13 %) as almost colorless solid. m.p. 76–77 °C; ¹H-NMR (400 MHz, CDCl₃): δ [ppm] = 1.43 (d, ³ $J_{H,H}$ = 7.49 Hz, 3 H, 4¹-CH₃), 1.91 (dd, ³ $J_{d\ H,H}$ = 7.33 Hz, J_d = 1.28 Hz, 3 H, 3²-CH₃), 3.55 (dq, J_d = 1.00 Hz, ³ $J_{q\ H,H}$ = 7.42 Hz, 1 H, 4-CH), 6.86 (dq, J_d = 2.15 Hz, ³ $J_{q\ H,H}$ = 7.34 Hz, 1 H, 3¹-CH), 7.82 (s, 0.5 H, NH), 8.05 (s, 0.5 H, NH); ¹³C-NMR (101 MHz, CDCl₃): δ [ppm] = 14.74 (C-3²), 15.55 (C-4¹), 38.49 (d, $J_{C,H}$ = 6.00 Hz, C-4), 132.71 (d, $J_{C,H}$ = 8.05 Hz, C-3), 135.24 (C-3¹), 169.11 (C-2), 177.89 (d, $J_{C,H}$ = 11.94 Hz, C-5); IR (KBr): $\tilde{\nu}$ = 3178, 3046, 2973, 2940, 2879, 1764,

1706, 1666, 1605, 1542, 1505, 1460, 1442, 1379, 1347, 1257, 1201, 1148, 1044, 1039, 948, 844, 776, 736, 709, 669, 627, 569, 439 cm^{-1} ; UV/Vis (methylene chloride): λ_{max} = 231, 313 nm (ϵ not determined); MS (EI, 35 $^{\circ}\text{C}$): m/z (%): 140 (24) $[\text{M}]^{+}$, 128 (1), 114 (2), 96 (63), 81 (2), 67 (100), 65 (9), 53 (47), 41 (27), 39 (41), 27 (21); HRMS (EI): m/z : calcd for $\text{C}_7\text{H}_9^{15}\text{NO}_2$ $[\text{M}]^{+}$: 140.060360; found: 140.060215.

(4E)-4-Ethylidene-3-methyl-5-thioxo(^{15}N)pyrrolidin-2-one (6): To a dispersion of Lawesson's reagent (0.70 g, 1.7 mmol) in 1,4-dioxane (5.4 ml) which was refluxed under argon a solution of **5** (0.24 g, 1.7 mmol) in 1,4-dioxane (1.6 ml) was added. The mixture was refluxed for additional 10 min and cooled on an ice bath immediately. By filtration through Celite 500 the precipitate was removed and repeatedly washed with 1,4-dioxane until it became colourless. Evaporation of the solvent yielded the crude product (0.95 g) which was purified by column chromatography with dichloromethane as eluent. The fraction containing the product was again purified by column chromatography with *n*-hexane and ethyl acetate (3:1, v/v) as eluent yielding the reaction product **6** (47 mg, 0.30 mmol, 18 %) as yellow solid. m.p. 105–106 $^{\circ}\text{C}$; ^1H -NMR (400 MHz, CDCl_3): δ [ppm] = 1.44 (d, $^3J_{\text{H,H}} = 7.53$ Hz, 3 H, 4^1-CH_3), 1.91 (dd, $^3J_{\text{d H,H}} = 7.48$ Hz, $J_{\text{d}} = 1.10$ Hz, 3 H, 3^2-CH_3), 3.39 (q, $^3J_{\text{H,H}} = 7.55$ Hz, 1 H, 4-CH), 7.12 (dq, $J_{\text{d}} = 2.02$ Hz, $^3J_{\text{q H,H}} = 7.47$ Hz, 1 H, 3^1-CH), 8.84 (s, 0.5 H, NH), 9.08 (s, 0.5 H, NH); ^{13}C -NMR (101 MHz, CDCl_3): δ [ppm] = 13.57 (C- 3^2), 13.60 (C- 4^1), 36.45 (d, $J_{\text{C,H}} = 7.91$ Hz, C-4), 135.75 (C- 3^1), 137.99 (C-3), 182.75 (C-5), 201.25 (C-2); IR (KBr): $\tilde{\nu} = 3226, 2963, 2927, 1752, 1733, 1664, 1444, 1380, 1316, 1263, 1247, 1223, 1188, 1027, 919, 860, 788, 761, 667, 618$ cm^{-1} ; UV/Vis (methylene chloride): λ_{max} = 258, 314 nm (ϵ not determined); MS (EI, 75 $^{\circ}\text{C}$): m/z (%): 156 (100) $[\text{M}]^{+}$, 141 (18), 127 (11), 112 (11), 97 (7), 94 (18), 84 (4), 77 (2), 67 (18), 60 (4), 53 (9), 39 (15), 39 (41), 27 (8); HRMS (EI): m/z : calcd for $\text{C}_7\text{H}_9^{15}\text{NOS}$ $[\text{M}]^{+}$: 156.037518; found: 156.037182.

Transformation of the ^{15}N -labeled ring A compound **6** to the full tetrapyrrole **10** followed published procedures (17, 18).

***tert*-Butyl 5-{2-(benzyloxy)-1-[(*E*)-3-ethylidene-4-methyl-5-oxo(¹⁵N)pyrrolidin-2-ylidene]-2-oxoethyl}-3-(3-methoxy-3-oxopropyl)-4-methyl-1*H*-pyrrole-2-carboxylate (7):** The crude product (0.25 g) was purified by column chromatography with *n*-pentane and ethyl acetate (gradient 1:0–7:3, v/v) as eluent. Subsequently, the fraction containing the product was purified by HPLC (column A) with methanol:water (5:1, v/v) as eluent, yielding the reaction product **7** (27 mg, 0.05 mmol, 17 %) as yellow colored crystals. m.p. 126–128 °C; ¹H-NMR (400 MHz, ¹H/¹H-COSY, CDCl₃): δ [ppm] = 1.34 (d, ³J_{H,H} = 7.42 Hz, 3 H, 2¹-CH₃), 1.55 (s, 9 H, CO₂C(CH₃)₃), 1.66 (d, ³J_{H,H} = 7.23 Hz, 3 H, 3²-CH₃), 1.78 (s, 3 H, 7¹-CH₃), 2.51 (t, ³J_{H,H} = 7.69 Hz, 2 H, 8²-CH₂), 2.94–3.08 (br m, 2 H, 8¹-CH₂), 3.11 (q, ³J_{H,H} = 7.14 Hz, 1 H, 2-CH), 3.63 (s, 3 H, CO₂CH₃), 5.03–5.31 (m, 1 H, 3¹-CH), 5.14 (br s, 2 H, 5²-CH₂), 7.17 (d, ³J_{H,H} = 6.68 Hz, 2 H, 5⁴-CH), 7.23–7.35 (m, 3 H, 5⁵-, 5⁶-CH), 8.43 (br s, 0.5 H, pyrrole-NH), 8.57 (br s, 0.5 H, pyrrole-NH), 10.70 (br s, 0.5 H, lactam-NH), 10.77 (br s, 0.5 H, lactam-NH); ¹³C-NMR (101 MHz, BB, ¹H/¹³C-COSY, CDCl₃): δ [ppm] = 9.12 (C-7¹), 15.86 (C-3²), 16.37 (C-2¹), 21.02 (C-8¹), 28.46 (CO₂C(CH₃)₃), 35.26 (C-8²), 37.90 (C-2), 51.39 (CO₂CH₃), 66.06 (C-5²), 80.90 (CO₂C(CH₃)₃), 119.12, 119.89, 125.49, 132.50, 133.63, 134.59 (C-3, -4, -6, -7, -8, -9), 127.31 (C-5⁴), 127.92 (C-3¹, -5⁶), 128.43 (C-5⁵), 136.17 (C-5³), 153.19 (C-5), 160.78 (CO₂C(CH₃)₃), 168.31 (C-5¹), 173.48 (CO₂CH₃), 177.80 (C-1); IR (KBr): $\tilde{\nu}$ = 3292, 3066, 2975, 2937, 1743, 1673, 1659, 1599, 1566, 1497, 1456, 1379, 1368, 1346, 1249, 1233, 1211, 1167, 1145, 1126, 1055, 949, 789, 735, 698, 624 cm⁻¹; UV/Vis [methanol/water (5:1, v/v)]: λ_{max} = 208, 234, 283 nm (ϵ not determined); MS (EI, 160 °C): *m/z* (%): 537 (74) [M]⁺, 481 (65), 436 (18), 390 (32), 372 (5), 346 (100), 331 (14), 286 (9), 254 (6), 228 (7), 135 (1), 107 (1), 91 (38), 65 (2), 41 (2); HRMS (ESI pos): *m/z*: calcd for C₃₀H₃₆N¹⁵NNaO₇ [M + Na]⁺: 560.238507; found: 560.238560.

[5-(*tert*-Butoxycarbonyl)-4-(3-methoxy-3-oxopropyl)-3-methyl-1*H*-pyrrol-2-yl]-2-[(*E*)-3-ethylidene-4-methyl-5-oxo(¹⁵N)pyrrolidin-2-ylidene]acetic acid (8): Purification of the crude product (38 mg) by HPLC (column A) with water and acetonitrile (gradient 49:1–0:1, v/v) as eluent yielded the reaction product **8** (8 mg, 0.02 mmol, 43 %) as yellow solid. Additionally, remaining **7** (6 mg, 0.01 mmol) could be recovered. m.p. 163–164 °C; ¹H-NMR (500 MHz, CDCl₃): δ [ppm] = 1.34–1.36 (m, 3 H,

2¹-CH₃), 1.56 (s, 9 H, CO₂C(CH₃)₃), 1.68 (d, ³J_{H,H} = 6.27 Hz, 3 H, 3²-CH₃), 1.81 (s, 1.5 H, 7¹-CH₃), 1.86 (s, 1.5 H, 7¹-CH₃), 1.96 (br s, 1 H, OH), 2.50–2.56 (m, 2 H, 8²-CH₂), 2.92–3.00 (m 1 H, 8¹-CH₂), 3.03–3.09 (m, 1 H, 8¹-CH₂), 3.14 (d, ³J_{H,H} = 6.33 Hz, 1 H, 2-CH), 3.65 (s, 3 H, CO₂CH₃), 5.27 (q, ³J_{H,H} = 6.28 Hz, 0.5 H, 3¹-CH), 5.34 (q, ³J_{H,H} = 6.28 Hz, 0.5 H, 3¹-CH), 8.73 (br s, 0.5 H, pyrrol-NH), 9.01 (br s, 0.5 H, pyrrol-NH), 10.67 (br s, 0.5 H, lactam-NH), 10.79 (br s, 0.5 H, lactam-NH); ¹³C-NMR (126 MHz, BB, DEPT 135, ¹H/¹³C-COSY, CDCl₃): δ [ppm] = 9.13 (C-7¹), 15.66 (C-3²), 16.38 (C-2¹), 20.90 (C-8¹), 28.18 (CO₂C(CH₃)₃), 35.02 (C-8²), 37.67 (C-2), 51.21 (CO₂CH₃), 81.00 (CO₂C(CH₃)₃), 133.23 (C-3¹), 119.04, 120.08, 125.28, 133.12, 134.23, 134.26 (C-3, -4, -6, -7, -8, -9), 153.29 (C-5), 160.92 (CO₂C(CH₃)₃), 170.66 (CO₂H), 173.42 (CO₂CH₃), 180.09 (C-1); IR (KBr): $\tilde{\nu}$ = 3448, 3314, 2977, 2935, 1739, 1670, 1605, 1565, 1455, 1382, 1368, 1337, 1248, 1210, 1165, 1147, 1126, 1051, 987, 948, 844, 795, 778, 746, 615, 457 cm⁻¹; UV/Vis [water/acetonitrile (49:1, v/v)]: λ_{max} = 235, 283 nm (ϵ not determined).

Ring A labeled (¹⁵N)-PCB dimethyl ester (9): The crude product (13 mg) was purified by column chromatography with dichloromethane and ethyl acetate (gradient 1:0–3:2, v/v) as eluent giving **9** (5 mg, 0.01 mmol, 46 %) as a bluish black solid. **9** was only characterized by its retention time compared with unlabeled PCB dimethyl ester.

Ring A labeled (¹⁵N)-PCB (10): The crude product (13 mg) was purified by HPLC (column B + C) with aqueous KH₂PO₄ buffer (7 mM, pH 7) and acetonitrile (7:3, v/v) as eluent. After removal of the acetonitrile under reduced pressure, the aqueous phase was extracted with chloroform (5 × 20 ml) and the combined organic phases were dried over Na₂SO₄. Subsequently, evaporation of the solvent under reduced pressure yielded **10** (3 mg, 0.005 mmol, 68 %) as a bluish black solid. **10** was characterized by its retention time in comparison to unlabeled PCB. UV/Vis (water): λ_{max} = 278, 363, 621 nm (ϵ not determined).

Biochemical Section

General Methods: Generation of *apo* Cph1Δ2 followed essentially formerly reported literature protocols (19). Sample treatment was at 4–8 °C, and spectroscopic measurements were performed under temperature controlled conditions at 20 °C. Assembly of the chromoprotein was accomplished by addition of **10** to an aqueous, buffered solution (50 mM Tris, 300 mM NaCl, 5 % glycerol, pH 8) of the *apo* Cph1Δ2 (8:1, n/n). Absorption spectra were recorded after the addition of **10** and purification of the chromoprotein over a nickel-containing resin. Measurements were done in the same buffer as used for the assembly reaction. After assembly, samples were handled under safe light (450–500 nm). They were repeatedly washed with the above buffer and afterwards with potassium phosphate buffer (50 mM potassium phosphate, 5 % glycerol, pH 7.8). Irradiation of the chromoprotein was performed with light from a 100 W slide projector bulb, equipped with an interference filter of 650 nm to achieve a maximum of P_{fr} form. The filter had a bandwidth of ± 5 nm and the sample was irradiated at a distance of 16 cm. Subsequently, concentration to 300 μ l yielded a highly viscous solution. UV/Vis: Shimadzu UV-2401 PC spectrometer. Data acquisition and treatment was done with the OriginPro 7.5 software.

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