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Synthesis of Selectively ^{13}C-Labelled Bilin Compounds

Yevgen Makhynya,[a] Zakir Hussain,[a] Tanja Bauschlicher,[a] Pascale Schwinte,[b] Friedrich Siebert,[b] and Wolfgang Gärtner*[a]

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A series of open-chain tetrapyrroles, each carrying one or more ^{13}C-labels in its three ring-bridging methine groups, were synthesized. These compounds – [5-, [10-, and [15-^{13}C]-phycocyanobilins (PCB), [10-^{13}C]-phytochromobilin, and [10,15-$^{13}C_2$]-PCB – were each obtained by a convergent route, starting with the four pyrrole building blocks, with the initial formation of the left and the right halves of the tetrapyrrole separately, followed by a final condensation of the two dipyrrole units to yield the target bilin compound. The ^{13}C-labels were all inserted as C_1- or C_2-units prior to the appropriate condensation. The substitution pattern of these bilins on ring A (ethylidene substituent at position 3) allows covalent attachment to the apoprotein of the plant photoreceptor phytochrome. The isotope shift produced by insertion of a ^{13}C-isotope is demonstrated in the FT-IR spectra of phytochrome-incorporated [10-^{13}C]-phytochromobilin.

Introduction

Biological photoreceptors gain their exceptional properties from intimate interactions between the chromophore and the protein moieties, which allow transmission of the dynamic, light-induced changes in the chromophore to the surrounding protein and the initiation of signal transduction. This is particularly the case for the covalently bound, highly flexible bilin chromophores of the red-/far-red-sensitive phytochromes, found ubiquitously in higher and lower plants and in many prokaryotes.[1–3] In addition to the investigation of the holoprotein by time-resolved absorption spectroscopy,[4] the light-induced conformational changes in the chromophore itself can be probed directly by several methods, such as vibrational (FT infra-red or Raman) spectroscopy or also by various NMR techniques. The highly complex spectra obtained from the parent states of these photoreceptors (P_rs), from their final photoproducts (P_{fr}s) or from potentially stabilizable intermediates are extremely difficult to interpret, which in the case of vibrational spectroscopy is due to the extended conjugation of the π system, giving rise to strongly coupled conformational modes.

For understanding of biological photoreceptors, and in particular for phytochromes, it is thus of particular importance to identify selectively certain positions that are assumed to be suggestive of the investigated conformational changes. The bilin chromophores of the phytochromes are known to undergo – in the instant light-induced process – a photoisomerization of the 15,16-double bond (connecting the pyrrole rings C and D; see Figure 1). This $Z \rightarrow E$ photoisomerization is assumed to be followed by various single-bond rotations and slight changes in the dihedral angles between the other rings, in order to compensate for steric hindrance to the surrounding protein after the double bond rotation.

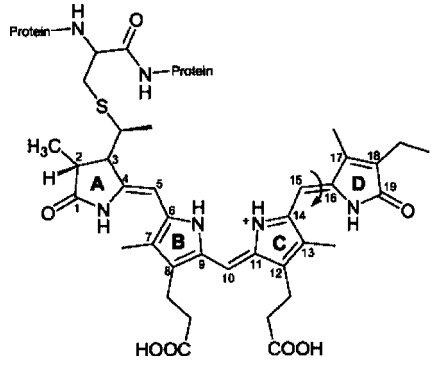


Figure 1. Structural formula of phycocyanobilin (**1**), covalently bound to cysteine321 of oat phytochrome. Attachment is accomplished through position 3′ in the A-ring, which in unbound form is part of an ethylidene substituent. The chromophore is shown in *ZZZ,asa* form. Phytochromobilin differs from PCB only in the substituent at position 18 (vinyl vs. ethyl). The photoisomerization of the 15–16 double bond is indicated by an arrow.

The synthesis of open-chain tetrapyrroles has been described for a large number of different derivatives, including some work on the introduction of stable isotopes.[5] We have recently presented a convergent synthesis, based on previously developed synthetic routes,[6] for an open-chain tetrapyrrole that, thanks to the separate synthesis of each ring, allows virtually every position of the tetrapyrrole to be ad-

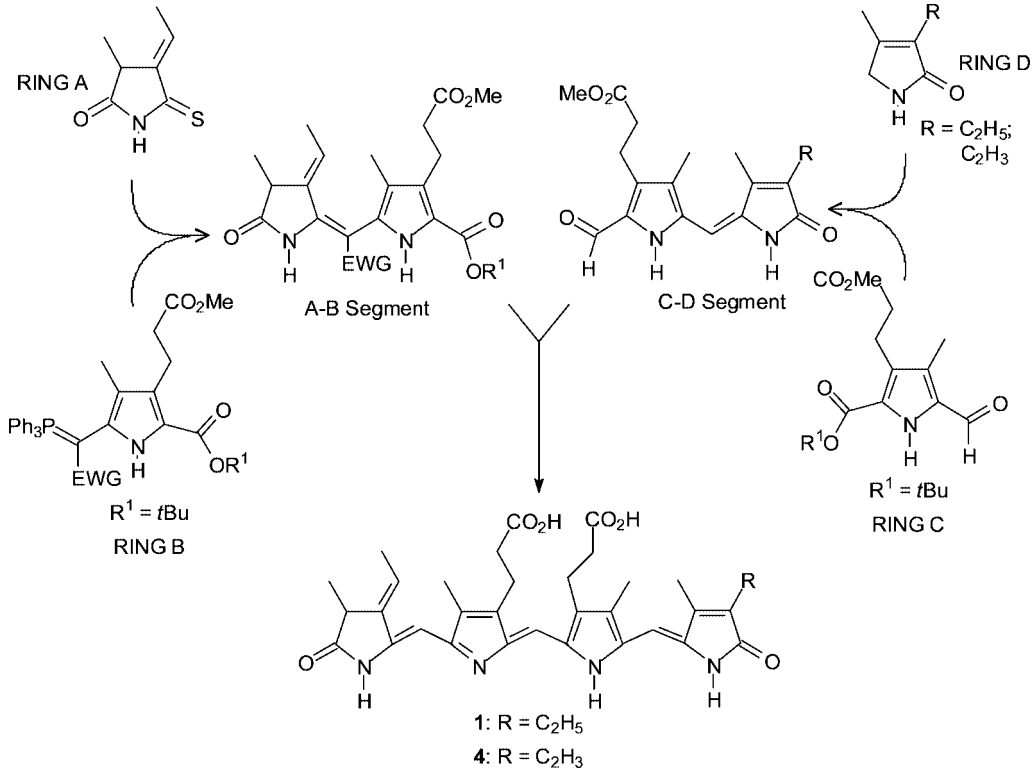
[a] Max-Planck-Institut für Bioanorganische Chemie, 45470 Mülheim, Germany
[b] Institut für Molekulare Medizin und Zellforschung, Sektion Biophysik, Hermann-Herderstr. 9, 79104 Freiburg, Germany

dressed for requested changes.[7] We now describe the selective introduction of ^{13}C-isotopes into the three methine bridge carbon atoms (positions 5, 10, and 15), together with one double labelling (positions 10 and 15), by application of this concept and demonstrate the initial vibrational (FTIR) spectra of these bilins after their incorporation into recombinant plant apo-phytochrome (phyA of oat). The same class of compounds allow detailed conformational analysis of the chromophore by ^{13}C and ^{15}N NMR spectroscopy, as the latter approach (^{15}N NMR) has recently demonstrated.[8]

Results and Discussion

Chemical Synthesis

Pyrroles to serve as precursors for rings A, B (which is identical to ring C) and D of bilin compounds were synthesized as recently described.[9] In the convergent synthesis described above, in which the left and right halves of a bilin were generated separately and finally condensed at its central position, C_1-synthons were used for the future positions 10 and 15 (see Scheme 1). The bridging position between rings A and B (position 5, Figure 1) was introduced as a C_2-unit.[10] Isotope labelling at positions 10 and 15 has in fact been described for several bilins,[5] thanks to the ready availability of the C_1-units, but no isotope introduction at position 5 had previously been accomplished. The condensation between rings A and B is one of the most challenging. A number of attempts have established that the Wittig reagent for condensation between the thioimide precursor of ring A and the ylide formed at ring B requires an electron-withdrawing substituent in order to prevent side reactions such as deprotonation of the pyrrole nitrogen atom in ring B, followed by electronic rearrangements.[11] On the other hand, though, since the ethylidene substituent on ring A is essential for covalent incorporation of a bilin into the plant photoreceptor phytochrome, any electronic rearrangement has be avoided during the condensation reaction. The compound usually employed is benzyl oxoacetate, formed by periodate-mediated oxidative splitting of dibenzyl tartrate. Benzyl oxoacetate was then attached to the free α-position of the B-ring pyrrole **8**, and the resulting hydroxy function was converted by chlorination/triphenylphosphane addition into a Wittig compound to connect rings A and B. Several alternative C_2-units for this condensation step in bilin synthesis have been proposed, amongst others acetonitrile, which would be excellently suited to support the reaction by virtue of its small size (see Figure 2). An attempted removal of the nitrile moiety from the C-5 position of rings A–B by reduction and retro-Mannich reaction was unsuccessful, however, so the very mild removal of a benzyl ester by hydrogenolysis and subsequent decarboxylation remains the most preferable procedure. For the purpose of introducing a ^{13}C-label the synthesis needs to be performed differently from routine methods, since benzyl oxoacetate is not available in isotope-labelled form, and so we employed [2-^{13}C]-labelled acetic acid as the C_2 building block. [2-^{13}C]-2-Bromoacetic acid was converted into the benzyl ester **6** and subsequently into its 2-iodo analogue **7**. The crucial



Scheme 1. Overview of the chemical synthesis of compounds **1**–**5**. EWG: electron-withdrawing group.

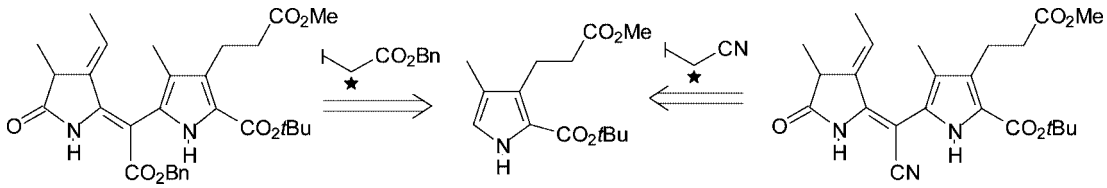
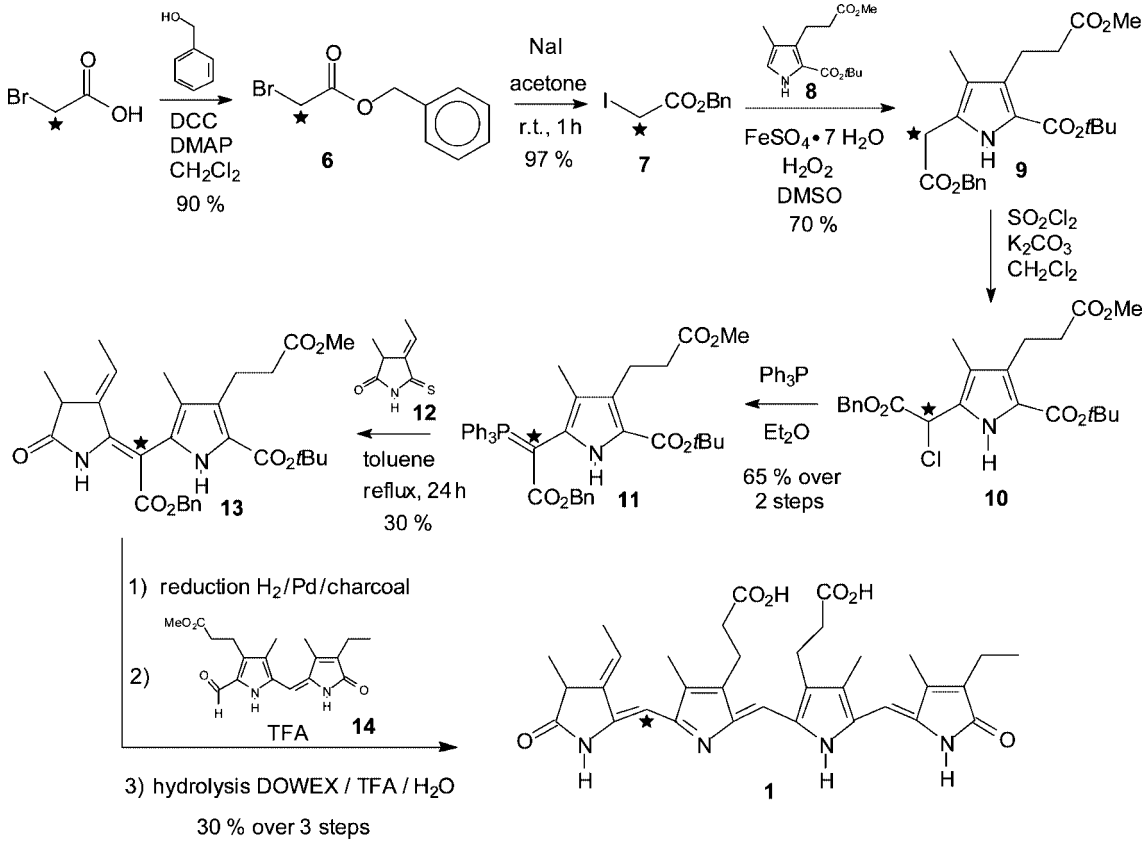


Figure 2. Retrosynthetic proposal for introduction of ^{13}C-label into position 5 of PCB (**1**).

step in this reaction sequence was then the alkylation of a pyrrole at a free α-position. Only two possible procedures were known – either by following a radical mechanism[12,13] or by performing the reaction in ionic liquids[14,15] – but both of these turned out to be inefficient for the synthesis of an all-carbon-substituted pyrrole, due to low yields. Nonetheless, we introduced benzyl [2-^{13}C]-2-iodoacetic acid by a radical reaction similar to that described in ref.[12,13] and succeeded in improving the methodology, making it applicable for the radical alkylation of mono-α-unsubstituted pyrroles in average to good yields. The synthesis of **9** in 70% isolated yield was thus accomplished (in addition, ca. 10% of starting material was recovered).

The resulting 5-{benzyloxycarbonyl([^{13}C]methyl)}pyrrole derivative **9** was chlorinated at the 5′-position (**10**) and then converted into the triphenylphosphonium chloride derivative and finally into the active ylide **11** by treatment with sodium carbonate. This Wittig reagent was condensed with thioimide **12** (A ring) without basic activation, to yield the [5-^{13}C]-dipyrrole compound **13**. This reaction is rather capricious and additionally has shown a strong light dependency. When it was performed under daylight illumination, only a 5% yield was obtained, whereas the high yield referred to above was obtained when the reaction vessel was protected from light. This "left half" of the target bilin was then condensed in the routine manner with the "right half" **14** to achieve the desired tetrapyrrole, still in its propionate dimethyl ester state. Hydrolysis with use of an ion exchange resin then yielded [5-^{13}C]-PCB **1** in an overall yield of 3.5% (relative to the starting amount of bromoacetic acid). The developed methodology for the introduction of a C-5 ^{13}C-label is a significant improvement in relation to previously published results[11] (which reported only a 25% yield for that particular condensation), and represents a versatile method for the construction of bilirubins, biliverdins and cyclic tetrapyrroles.

The synthesis of [10-, [15- and [10,15-$^{13}C_2$]-PCB (**2**, **3** and **4**) followed the routine synthesis scheme (Scheme 1). Insertion of a ^{13}C atom at position 10 was performed by formylation (trimethyl orthoformate, formyl ^{13}C) of position



Scheme 2. Synthesis of 5-^{13}C PCB. Bn: benzyl.

11 of the dipyrrole **14** constituting the "right half" (numbering according to open-chain tetrapyrroles; see Figure 1), followed by condensation with the "left half" and final saponification of the dimethyl esters at rings B and C. Insertion of a ^{13}C label at position 15 was accomplished by formylation of the α-free pyrrole **8** with ^{13}C-labelled DMF (formyl ^{13}C) and $POCl_3$, followed by condensation with the D-ring building block. This compound was then formylated in a routine way, to give the "right half" of the desired tetrapyrrole, and this was treated with the "left half" as described above. The tetrapyrrole compound was again finally saponified, yielding [15-^{13}C]-PCB. A combination of the described isotope label insertion reactions also finally yielded [10,15-$^{13}C_2$]-labelled PCB (Scheme 2).

In the synthesis of **5**, the left half of the target tetrapyrrole was prepared as described in the cases of **1**–**4**. The right half, though, unlike in the procedure described for **1**–**4**, was obtained by the cleavage of biliverdin IXa diethyl ester with thiobarbituric acid and subsequent formylation of the C-D half with $^{13}C_1$-trimethyl orthoformate.

All five isotope-labelled bilin compounds **1**–**5** were obtained in mg amounts and were identified by their spectral properties, which were identical to those of their unlabelled counterparts. The insertion of single ^{13}C labels – or, in the case of compound **4**, a double ^{13}C label – was unambiguously verified by their mass spectra. Bilins prepared in the manner described here are always racemic at position 2 in the A-ring. However, since assembly with the apoprotein is always performed in the presence of an excess of chromophore, due to its instability under the incubation conditions, no special effort to generate an enantiomerically pure tetrapyrrole was made.

After HPLC purification, the labelled PCBs were incubated with the recombinantly expressed apo-protein of oat phyA (N-terminal 65 kDa half). In each case the expected P_r- and P_{fr}-specific absorption spectra were obtained, indicating that this material could be used for spectroscopic investigations. The effect of isotope incorporation can clearly be seen in a P_r/P_{fr} FTIR difference spectrum recorded with a 10-^{13}C-PΦB incorporated into apo-phyA (Figure 3). Difference spectra are commonly presented as photoproduct minus parent state (i.e., $P_{fr} - P_r$ state). As can be seen in the difference spectrum, most difference bands remain at identical positions, but others (indicated by small arrows) exhibit characteristic shifts in the band position. Even with the very complex structure of the spectrum, one can clearly identify band patterns that can be ascribed to the single incorporated isotope label. A detailed analysis of infrared and of Raman spectra, also including spectra of the various photoprocess intermediates, should allow a deeper view into the conformational changes that take place during the P_r to P_{fr} and P_{fr} to P_r conversion of phytochrome and will be described elsewhere.

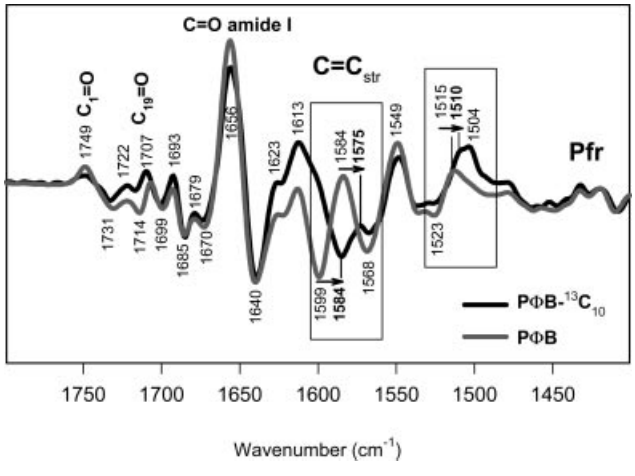


Figure 3. FTIR difference spectrum ($P_{fr} - P_r$) of 10-^{13}C-labelled PΦB, incorporated into apo phyA of oat (65 kDa N-terminal domain). Positive bands are assigned to the photoproduct (P_{fr}) whereas negative bands are related to the P_r form. Changes due to the isotope shift are indicated by arrows.

Experimental Section

Abbreviations: PCB: phycocyanobilin, P_r, P_{fr}: red-, far-red absorbing forms of phytochrome, PΦB, phytochromobilin.

Chemical Synthesis: For an overview on the synthetic routes see Scheme 1. IUPAC nomenclature is only used for the isolated ring compounds.

Benzyl [2-^{13}C]-2-Bromoacetate (6):[16] [2-^{13}C]-2-Bromoacetic acid (1 g, 7.15 mmol, 99% isotope purity, Aldrich, Germany) and benzyl alcohol (0.79 g, 7.3 mmol) were dissolved in dichloromethane (30 mL), and dicyclohexyldicarbodiimide (DCC; 1.51 g, 7.33 mmol) was added, followed by a catalytic amount (75 mg, 0.6 mmol) of 4-(dimethylamino)pyridine (DMAP). The mixture was stirred overnight and finally chromatographed on a silica gel column with dichloromethane. Concentration in vacuo gave the corresponding benzyl ester **6** as a colourless liquid (1.48 g, 90%).

Benzyl [2-^{13}C]-2-Iodoacetate (7): Benzyl [2-^{13}C]-2-bromoacetate (1.48 g, 6.47 mmol) was dissolved in acetone (20 mL). NaI (1.32 g, 8.8 mmol) was added and the mixture was stirred at room temperature for 1 h, during which a voluminous white precipitate formed. The solvent was evaporated, and the remaining solid was separated between water and ether. The ether phase was separated, dried with Na_2SO_4 and concentrated in vacuo to yield benzyl [2-^{13}C]-2-iodoacetate (**7**, 1.72 g, 97%) as a yellow oil. ^1H NMR (400 MHz, $CDCl_3$): δ = 7.37 (m, 5 H, CH-benzyl), 5.16 (s, 2 H, CH_2-benzyl), 3.70 (d, J = 153 Hz, 2 H, CH_2-2) ppm.

***tert*-Butyl 5-{Benzyloxycarbonyl([^{13}C]methyl)}-3-[2-(methoxycarbonyl)ethyl]-4-methyl-1*H*-pyrrole-2-carboxylate (9):** One third of the calculated amount (0.2 g, 2.06 mmol) of H_2O_2 (35% solution in water, the whole amount being 6.17 mmol, 0.6 g) was added dropwise to a stirred solution of α-free pyrrole **8** (1.1 g, 4.11 mmol), benzyl [2-^{13}C]-2-iodoacetate (0.57 g, 2.07 mmol; 1/3 of the total amount) and $FeSO_4 \cdot 7H_2O$ (0.23 g, 0.82 mmol; 1/3 of the total amount) in DMSO (40 mL). The reaction was slightly exothermic, so a water bath was used to keep the reaction at room temperature. After the mixture had been stirred for one hour, further portions (again 1/3 of the total amount) of benzyl [2-^{13}C]-2-iodoacetate, $FeSO_4 \cdot 7H_2O$ and H_2O_2 were added. Finally, after the mixture had been allowed to react for another hour, the last third of the reagents was added. The colour of the reaction mixture changed from light yellow to brown-red during the addition of H_2O_2, but turned back to yellow during the course of the reaction. After the mixture had been stirred for an additional 3 h, the reaction was quenched by

the addition of a saturated brine solution and subsequently extracted with diethyl ether (3 × 50 mL). The combined organic phases were washed with brine, dried with Na_2SO_4 and concentrated in vacuo. Column chromatography with a pentane/diethyl ether gradient (2:1 → 1:1) as eluent resulted in the target alkylated pyrrole (**9**, 1.2 g, 70% isolated yield), initially obtained as a very viscous yellow oil, which slowly crystallized to yield light yellow crystals. Additionally, 135 mg (12% of the starting amount) of the α-free pyrrole **8** was recovered. ^1H NMR (400 MHz, $CDCl_3$, $^1H/^1H$-COSY): δ = 9.08 (s, 1 H, NH), 7.29–7.37 (m, 5 H, CH-phenyl), 5.14 (s, 2 H, H_2C-benzyl), 3.64 (s, 3 H, CO_2Me), 3.60 (d, J = 130 Hz, 2 H, H_2C-5^1), 2.97 (m, 2 H, CH_2-3^1), 2.50 (m, 2 H, CH_2-3^2), 1.92 (s, 3 H, CH_3-4^1), 1.53 [s, 9 H, 3 CH_3 (*t* Bu)] ppm. ^{13}C NMR (100 MHz, $CDCl_3$, BB, DEPT, $^1H/^{13}C$-COSY): δ = 173.70 (Cq, CO_2Me), 169.59 (Cq, CO_2Bn), 160.57 (Cq, CO_2tBu), 135.40 (Cq, Ar), 128.62 (CH, Ar) 128.52 (d, J = 2.5 Hz, Cq-3), 128.45 (CH, Ar), 128.29 (CH, Ar), 123.58 (Cq, C-5), 119.43 (d, J = 1.7 Hz, Cq, C-2), 117.72 (d, J = 4 Hz, Cq, C-4), 80,63 (Cq, C-*t*Bu), 67.03 (CH_2-benzyl), 51.41 (CH_3, OMe), 34.98 (CH_2, C-3^2), 31.56 (m, CH_2, C-5^1), 28.43 (CH_3, *t*Bu), 20.76 (CH_2, C-3^1), 8.56 (CH_3, C-4^1) ppm. MS (EI, 130 °C): *m*/*z* (%) = 416 (43) $[M]^+$ ($C_{22}{}^{13}CH_{29},NO_6$), 360 (54) $[M - C_4H_8(tBu)]^+$, 343 (5), 329 (14), 225 (100) $[M - C_4H_8(tBu) - CO_2Bn]^+$, 207 (12), 193 (19), 181 (16), 165 (11), 91 [77, $C_7H_7^+$ (tropylium ion)], 57 [6, $C_4H_9^+(tBu)$], 41 (3). HRMS (ESI-pos): $C_{22}{}^{13}CH_{29}NNaO_6$ [M + Na]: theor: 439.192064; found: 439.192369.

***tert*-Butyl 5-{Benzyloxycarbonylchloro([^{13}C]methyl)}-3-[2-(methoxycarbonyl)ethyl]-4-methyl-1*H*-pyrrole-2-carboxylate (10):** The labelled pyrrole **9** from the previous step (503.2 mg, 1.2 mmol) was added to a suspension of anhydrous K_2CO_3 (1.66 g, 12 mmol) in dichloromethane (50 mL) and the system was cooled to –78 °C with a dry ice/acetone bath. SO_2Cl_2 (162.3 mg, 1.2 mmol) was added dropwise at this temperature and the reaction mixture was allowed to stir for a further 3 h, the temperature being maintained below –70 °C. Afterwards the cooling was removed and the reaction mixture was allowed to warm to room temperature. The colour of the mixture changed from light yellow to yellow-brown. Potassium carbonate was filtered off and the solvent was removed in vacuo. The crude product was transferred to a next step without further purification.

[5-^{13}C]-Labelled A–B Ring Dipyrrole (13): The crude product **10** from the previous step was dissolved in ether (15 mL) and a solution of triphenylphosphane (349 mg, 1.33 mmol) in ether (6 mL) was added dropwise. The reaction mixture was stirred at room temperature overnight. After this stirring, a voluminous white precipitate had formed, and this was removed by filtration. A second portion was obtained from the mother liquid. The combined portions of the phosphonium salt were dissolved in dichloromethane and washed with saturated $NaHCO_3$, and the organic phase was dried with sodium sulfate and concentrated to give a yellow, very viscous oil. This oil was dried in vacuo to give the target phosphorus ylide **11** as a yellow voluminous foam (527 mg, 65%). This foam had a UV spectrum and an R_f value identical to those of the unlabelled compound,[10,11] so no further analysis was performed.

The coupling to yield **13** was performed in the dark and under argon. The phosphorus ylide **11** (227 mg, 0.335 mmol; ring B) and the thioimide **12** (52 mg, 0.335 mol; ring A, prepared by the literature procedure[17]) were dissolved in dry toluene (30 mL). This reaction mixture was heated at reflux for 24 h and the colour changed from yellow to reddish. The solvent was evaporated and the resulting residue was purified by HPLC (pentane/ethyl acetate, 2:1). After removal of the solvents and further drying in vacuo, the target benzyl ester **13** was obtained as a yellow foam (53 mg, 30%). ^1H NMR (400 MHz, $CDCl_3$, $^1H/^1H$-COSY): δ = 10.80 (s, 0.5 H, NH), 10.70 (s, 0.5 H, NH), 9.12 (br s,0.5 H, NH), 8.90 (br s, 0.5 H, NH), 7.14–7.24 (m, 5 H, CH phenyl), 5.29 (q, J = 6.93 Hz, 0.6 H, HC-3^1), 5.19 (m, 0.4 H, HC-3^1), 5.00–5.16 (m, 2 H, benzyl CH_2), 3.62 (s, 3 H, CO_2Me), 3.09 (m, 1 H, HC-2), 2.99 (m, 2 H, CH_2-8^1), 2.50 (m, 2 H, CH_2-8^2), 1.77 (s, 3 H, CH_3-7^1), 1.64 (m, 3 H, CH_3-3^2), 1.50 (s, 9 H, CO_2tBu), 1.31 (m, 3 H, $CH_{3\ -21}$) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, BB, DEPT, $^1H/^{13}C$-COSY): δ = 177.86 (Cq, C-1), 173.48 (Cq, CO_2Me), 168.70 (Cq, CO_2Bn), 161.00 (d, J = 12.7 Hz, Cq, CO_2tBu), 152.88 (dd, J = 81.4, 25.8 Hz, Cq, C-5), 136.14 (Cq, Ar), 134.36 (d, J = 16.4 Hz, Cq, pyrrole), 133.68 (Cq, pyrrole), 132.52 (Cq, pyrrole), 128.33 (CH, Ar), 127.79 (CH, Ar), 127.21 (CH, C-3^1), 127.11 (CH, Ar), 125.60 (dd, J = 69.7, 20.4 Hz, Cq, pyrrole), 119.86 (Cq, pyrrole), 118.90 (Cq, pyrrole), 91.92 (m, enriched *meso*), 80.84 (Cq, *t*Bu), 65.92 (CH_2, benzyl), 51.38 (CH_3, OMe), 37.82 (d, J = 8.9 Hz, CH, C-2), 34.24 (CH_2,C-8^2), 28.30 (3 × CH_3, *t*Bu), 21.06 (CH_2,C-8^1), 16.40 (d, J = 36.7 Hz, CH_3,C-2^1) 15.97 (d, J = 29.5 Hz, CH_3,C-3^2), 9.04 (CH_3,C -7^1) ppm. MS (EI, 165 °C): *m*/*z* (%) = 537 (70) $[M]^+$ ($C_{29}{}^{13}CH_{36},N_2O_7$), 481 (55) $[M - C_4H_8(tBu)]^+$, 466 (12), 436 (10), 390 (23), 346 (100) $[M - C_4H_8(tBu) - CO_2Bn]^+$, 286 (8), 228 (6), 91 [54, $C_7H_7^+$ (tropylium ion)], 57 [5, $C_4H_9^+(tBu)$], 41 (3) HRMS (ESI-pos): $C_{29}{}^{13}CH_{36}N_2NaO_7$ [M + Na]: theor: 560.244827; found: 560.244888.

Removal of Benzyl Ester from 13: In the dark and under argon, the benzyl ester **13** (53 mg, 0.097 mmol) was dissolved in absolute THF (7 mL) and palladium on charcoal (10%, 34 mg, 0.032 mmol) was added without stirring. Vacuum was applied, until the solvent started to boil, and the reaction vessel was flushed with hydrogen. This procedure was repeated five times. The reaction mixture was then stirred at ambient temperature for 1.5 h (the reaction was monitored by TLC). Finally, the mixture was filtered through a double paper filter (blue band) and the filter was washed with THF. The combined organic phases were concentrated and the residue was subjected to HPLC separation (water/acetonitrile/TFA, gradient from 97.95:2:0.05 to 0:99.95:0.05) to give the corresponding acid (17.4 mg, 40% isolated yield, 75% considering recovered starting material). Some benzyl ester **13** (24 mg, 45% from the starting material) was also recovered.

Synthesis of [5-^{13}C]-Labelled Phycocyanobilin Dimethyl Ester (1-Dimethyl Ester): The coupling was performed in the dark and under constant argon flow. The A–B rings compound (17.4 mg, 0.039 mmol), the left half of the target molecule, was dissolved in TFA (1 mL) and the system was stirred for 30 min at room temperature. The reaction mixture was cooled to –15 °C with an acetone/dry ice bath. The right half (C–D rings **14**, prepared and purified by the already published procedure,[18] 13.5 mg, 0.041 mmol) was dissolved in TFA (4 mL) and added dropwise. The reaction was allowed to proceed for an additional 8 h, and the temperature was kept between –10 and –15 °C. Finally, methanol (2 mL) was added at this temperature. The cooling bath was removed and the mixture was stirred for another 30 min. During this time the mixture warmed to room temperature and a colour change from reddish to dark blue was observed. The solvent was removed carefully under a strong argon flow and the resulting residue was dissolved in dichloromethane (30 mL). The organic phase was washed with diluted $NaHCO_3$ (30 mL) and subsequently with water until neutral pH, dried and concentrated under reduced pressure. The remaining residue was purified by HPLC (methanol/water 5:1). After removal of the solvent, the [5-^{13}C]-labelled phycocyanobilin dimethyl ester was obtained as a blue solid (12.2 mg, 55%). This

product was then transferred to the next step without any characterisation.

[5-^{13}C]-Labelled Phycocyanobilin (1): [5-^{13}C]-Labelled phycocyanobilin dimethyl ester (12.2 mg, 0.02 mmol) was dissolved, in the dark and under argon, in a mixture of trifluoroacetic acid and water [1:1 (v:v), 30 mL], and acidic ion exchange resin DOWEX (12.3 g) was added. The resin had been washed extensively with water and dried in air prior to its addition. This suspension was stirred at room temperature for 64 h, and the resin was finally filtered off (ceramic filter). The residue was washed successively on a filter paper, alternately with water (40 mL, five times each) and with a chloroform/methanol mixture (40 mL, 49:1 v/v). The aqueous phase was separated and extracted with a chloroform/methanol (49:1 mixture, four times, 40 mL each). The combined organic phases were washed with brine until pH 6 and dried with Na_2SO_4. Evaporation of the solvents resulted in the crude product as a dark blue solid that was further purified by HPLC (7 mM KH_2PO_4 buffer, pH 7, acetonitrile; gradient from 7:3 to 1:4). After removal of methanol, chloroform (30 mL) and water (30 mL) were added to the residue and the phases were separated. The water phase was extracted with chloroform until the aqueous layer remained colourless, and the combined organic phases were then dried with Na_2SO_4. After filtration, the Na_2SO_4 cake was washed extensively with a chloroform/methanol mixture (4:1 v/v), until the cake was nearly colourless. Evaporation of the solvent resulted in the [5-^{13}C]-labelled phycocyanobilin **1** (8 mg, 70%) as a dark blue solid. Qualitative HPLC analysis revealed the product to be homogeneous. Partial NMR: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.96 (d, J = 158.67 Hz, CH-5) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ = 88.92 (CH, C-5) ppm. HRMS (ESI – pos.): $C_{32}{}^{13}CH_{39}N_4O_6$ [M + H]: theor: 588.289767; found: 588.289637.

[10-^{13}C]-Labelled Phycocyanobilin (2): The α-free pyrrole was formylated with DMF and $POCl_3$ to afford the corresponding aldehyde, which was condensed with ring D (synthesized by the published procedure[19]) to give the right half (C–D ring building block). This was again formylated, this time with trimethyl orthoformate (formyl ^{13}C) in trifluoroacetic acid as described for phytochromobilin.[18] The latter compound was condensed with the left half (A–B ring building block) in the routine manner to give the [10-^{13}C]-labelled PCB as its dimethyl ester. Hydrolysis afforded the [10-^{13}C]-labelled phycocyanobilin in 20% yield (for the last two steps).

[15-^{13}C]-Labelled Phycocyanobilin (3): The α-free pyrrole was formylated with labelled DMF (^{13}C-formyl) and $POCl_3$ to afford the labelled aldehyde, which was condensed with the ring D as described for [10-^{13}C]-labelled PCB (**2**). Further formylation and subsequent coupling with the A–B ring building block, followed by hydrolysis, resulted in the [15-^{13}C]-labelled PCB (24% yield for the last two steps). HRMS (ESI – pos. + neg.): $C_{32}{}^{13}CH_{39}N_4O_6$ [M + H]: 588, ^{13}C incorporation > 95%.

[10,15-$^{13}C_2$]-Labelled Phycocyanobilin (4): The α-free pyrrole was formylated with labelled DMF (^{13}C-formyl) and $POCl_3$ to give the labelled aldehyde, which was condensed with ring D and formylated with trimethyl orthoformate (formyl ^{13}C) in trifluoroacetic acid. Finally, this C–D ring building block was condensed with the A–B ring segment to afford [10,15-$^{13}C_2$]-labelled phycocyanobilin dimethyl ester in 35% yield. Hydrolysis gave [10,15-$^{13}C_2$]-labelled phycocyanobilin in 80% yield. HRMS (ESI – pos. + neg.): $C_{31}{}^{13}C_2H_{39}N_4O_6$ [M + H]: 589, ^{13}C incorporation > 95%.

Synthesis of [10-^{13}C]-Labelled Phytochromobilin (5): Biliverdin IX*a* diethyl ester (315 mg, 0.49 mmol) was treated with thiobarbituric acid (95.4 mg, 0.66 mmol) in ethyl acetate (640 mL) as described by Lindner,[9] to yield the right and the left halves of the desired molecule separately. The C–D ring building block (32 mg, 97.4 μmol) was formylated with trimethyl orthoformate (formyl ^{13}C, 1.52 mmol) in trifluoroacetic acid (3 mL) and condensed with the "left half", to yield [10-^{13}C]-phytochromobilin as the methyl ethyl ester in 45% yield.[9] [10-^{13}C]-Phytochromobilin was obtained upon ester hydrolysis, as described for the PCB counterparts, in 89% yield. HRMS (ESI – pos. + neg.): $C_{32}{}^{13}CH_{36}N_4O_6$ [M – H]: 584, ^{13}C incorporation > 95%.

Biochemical Procedures: The N-terminal half of apo-phytochrome of oat (*Avena sativa*), spanning positions 1–595, was heterologously expressed in *Hansenula polymorpha*. A C-terminally attached His_6-tag allowed affinity purification on Ni-NTA (Serva, Heidelberg) as described.[20] Assembly with ^{13}C-labelled PCBs (**1**–**5**) was performed with the crude cell lysate prior to purification. The isolated chromoprotein was characterized by SDS-PAGE. The P_r and P_{fr} states were produced by far-red and red irradiation of the chromoproteins, respectively. The extent of photoconversion was followed by UV/Vis absorption spectroscopy (Shimadzu UV2401-PC).

FTIR Spectroscopy: For the FTIR measurements, the recently described sandwich cuvette was used.[21] All other parameters – sample preparation, measuring conditions such as in-situ illumination, number of scans, and spectral resolution, data collection and treatment – were essentially identical to those recently described.[22]

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