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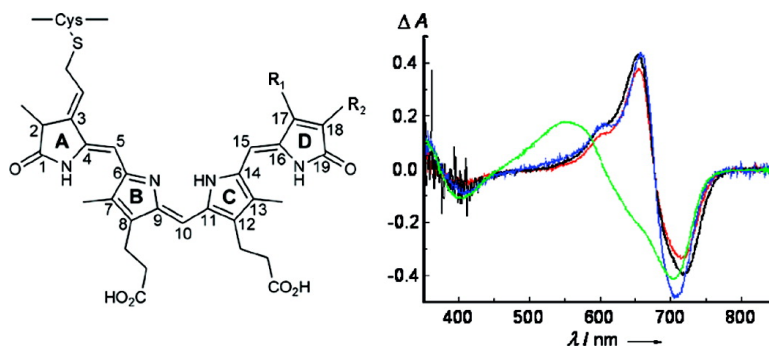# New Open-Chain Tetrapyrroles as Chromophores in the Plant Photoreceptor Phytochrome

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A R T I C L E S

New Open-Chain Tetrapyrroles as Chromophores in the Plant Photoreceptor Phytochrome

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Abstract: A series of six open-chain tetrapyrroles has been synthesized and used as chromophores for the plant photoreceptor protein phytochrome. The novel chromophores vary in the size of substituents 17 and 18 at ring D. This ring undergoes maximal conformational change upon light excitation ($Z \rightarrow E$ photoisomerization of the 15,16-double bond). Instead of methyl and vinyl substituents (positions 17, 18) as present in the native chromophore phytochromobilin, dimethyl, methyl and isopropyl, methyl and *tert*-butyl, ethyl and methyl, vinyl and methyl, and isopropyl and methyl substituents have been generated. All novel chromophores assemble with the apoprotein. The obtained chromoproteins show hypsochromic shifts of the absorbance maxima by 10 nm maximally, compared to the native pigment, except for the 17-isopropyl-18-methyl-substituted compound which showed a 100 nm hypsochromic shift of selectively the P_r form. The assembly kinetics were slowed down in correlation to the increasing size of the substituents, with stronger effects for modified substituents at position 17. The thermal stability of the photoinduced P_{fr} form for the 18-isopropyl and the 18-*tert* butyl substituents was even greater than that of the native pigments. Those chromophores carrying substituents at position 17 larger than the methyl group (ethyl and isopropyl) showed a very low stability of the respective P_{fr} forms. Time-resolved detection of the P_r to P_{fr} conversion (laser-induced flash photolysis) revealed a slower formation of the P_{fr} form for those chromophores carrying larger substituents at position 18, whereas the rise and decay kinetics of the early intermediates are only moderately changed. Introduction of larger substituents at position 17 (ethyl, vinyl, and isopropyl) causes drastic changes in the kinetics; in particular the formation of the first thermally stable intermediate, I_{700}, is significantly slowed, making a detection of its rise possible.

1. Introduction

Open-chain tetrapyrroles, also called bilins, are important biological components which are formed during the catabolism of heme components. Functionally, they constitute the light-absorbing components in the antenna systems of many cyano-bacteria and several algae.[1] Furthermore, they serve as chromophores in the plant and bacterial phytochromes, which act as photosensors.[2] Besides the linear arrangement of four pyrrole rings, connected via methine or methylene bridges, the substitution pattern in this class of compounds is very heterogeneous (according to their biological origin). Several tetrapyrrole compounds can be obtained from natural sources, and to some extent their chemical properties allow a certain variation of the substitution pattern.[3-5] Yet, the availability of recombinant, tetrapyrrole-binding proteins has increased the demand for a broader structural variation of these substances in order to analyze in greater detail the interactions between a given or recombinant protein binding site and the incorporated tetra-pyrrole compound. Apart from the classical synthetic approach,[6] a number of partial and total syntheses have been reported during recent years. Still, a versatile synthetic route for the generation of this class of natural compounds, allowing facile modification of the substituents in any of the four pyrrole rings and also in the connecting methine bridges is a strong demand.

We reported previously on the synthesis of several novel, structurally modified bilins in conjunction with investigations on chromophore–protein interactions in the plant photoreceptor phytochrome.[5] In this chromoprotein, the tetrapyrrole chromophore is bound covalently to the protein via a thioether, formed between the thiol group of a cysteinyl residue of the protein and the position 3′ of the originally present ethylidene group in the A ring of the chromophore (Figure 1; it should been mentioned that in some bacterial phytochromes biliverdin serves as chromophore, which then is bound to the protein via its 3″-position). Once incorporated into the apo-phytochrome binding site, the chromophore, upon light absorption, undergoes a photochromic reaction (photoisomerization of the 15,16-double bond) between two thermally stable states, P_r and P_{fr} (r, fr =

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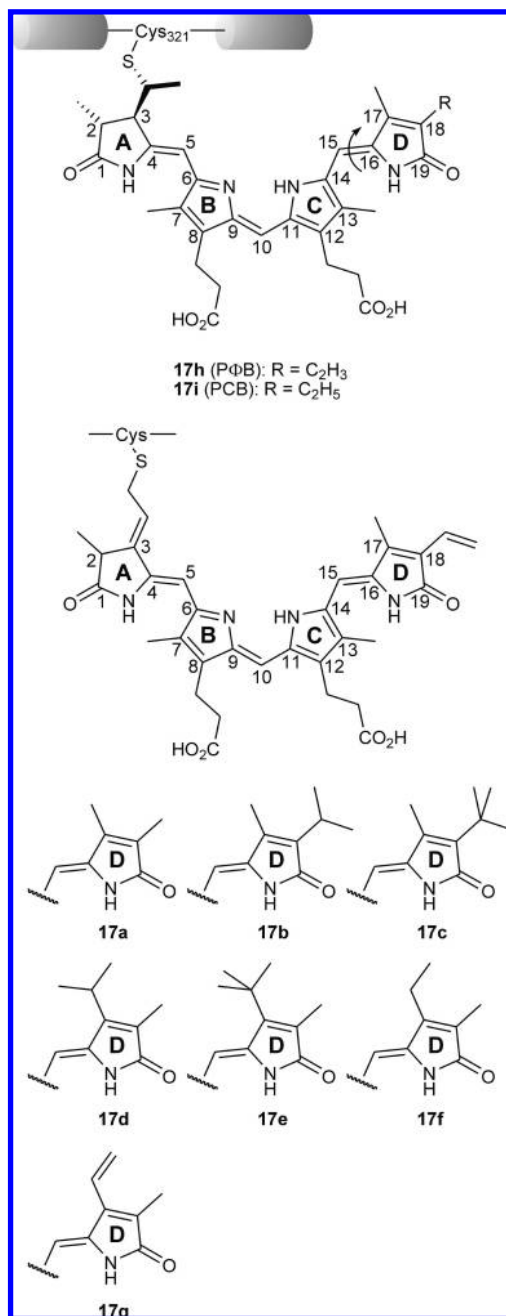
Figure 1. Structural formulas of naturally occurring bilins, found as chromophores in plant and bacterial phytochromes. Top: Phytochromobilin (PΦB) is shown in the protein-bound form, attached to the thiol group of a cysteine residue via its 3′-position. Phycocyanobilin (PCB) differs from PΦB by an ethyl instead of vinyl substituent at position 18 (R = vinyl or ethyl, respectively). Phycoerythrobilin (PEB, structure not shown in detail), carrying a saturated bridge between rings C and D, has been used as a nonisomerizing, strongly fluorescent chromophore in several recombinant phytochromes.[26] Middle: Biliverdin IXα (BV) was found in several bacterial phytochromes, covalently bound via its 3″-position.[34−38] Bottom: D-rings of novel synthesized bilin derivatives from this study. Compound numbers refer to Scheme 3. Compound **17e** could not be performed because of intramolecular steric hindrance. Only the building block, showing this substitution pattern, was synthesized.

red, far-red absorbing forms, λ_{max}: 667 and 730 nm, respectively; for details on the photochemistry of phytochromes, see refs 7, 8). In particular the change of the spectroscopic parameters (a strong increase of the extinction coefficient for the long-wavelength absorption band on the expense of the 340 nm band of the free

chromophore) and the strong shift of the absorption maximum of especially the P_{fr} state of phytochrome has evoked a number of chemical approaches employing model compounds[9,10] but also spectroscopic and theoretical attempts to understand this phenomenon.[11] Additional information on the structure of the P_r state was provided by a recently published crystal structure of the chromophore binding domain of a bacterial phytochrome from *Deinococcus radiodurans*,[12] which, however, carries a covalently bound chromophore biliverdin IXα (BV) instead of phytochromo- or phycocyanobilin (PΦB (**17h**) or PCB (**17i**), respectively; for structural details, see Figure 1). Since the overall structure of BV is very similar to that of PΦB or PCB, a three-dimensional conformation of the latter two bilins similar to that of BV might be assumed within the protein cavity, even despite the fact that BV is bound via its 3″-position (employing a different cysteine than that at the canonical binding site), whereas PΦB or PCB are bound at position 3′ to the protein. Unfortunately, the crystallized chromophore-binding phytochrome domain from *D. radiodurans* is impaired in the formation of a stable P_{fr} state, making information on the photochemical process from other methods still desirable. Since ring D undergoes the greatest conformational changes upon light activation (photoisomerization of the double bond between rings C and D), any change in its structure or substitution pattern is of strong interest. Here, we describe the total synthesis of linear tetrapyrroles of the PΦB/PCB type with special emphasis on modifications of the D-ring substituents. According to the covalent attachment of the chromophores in the plant and in most cyanobacterial phytochromes at 3′-position, the target compounds were designed with an ethylidene group at position 3 (in ring A), which upon nucleophilic attack of a cysteine thiol group yields the covalent attachment to the protein. Also, the tetrapyrrole compound requires both propionic acid side chains in the free-acid form,[13] which is essential for a functional phytochrome chromophore. The above-mentioned protein crystal structure rationalized the need of free propionic acids in the bilin from the finding of an electrostatic interaction between B/C-ring propionates and a nearby arginine residue. In particular this latter demand represents a remarkable synthetic challenge, since most classical routes for the *de novo* synthesis of open-chain tetrapyrroles aim at the more stable propionic acid dimethyl esters as target molecules. We report here the synthesis of a series of bilins, carrying substituents of different size at positions 17 and 18 at the D-ring, their incorporation into the recombinant apo-phytochrome from oats, and the spectral and kinetic properties of the assembled chromoproteins.

2. Results

Chemical Synthesis. The synthesis of open-chain tetrapyrroles follows a well established protocol,[6] although in recent times several alternative methods have been presented. However, only a limited number of research groups address the total synthesis of tetrapyrroles.[14−17] Activities concentrate on more conve-

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Scheme 1. Synthesis of 1*H*-Pyrrolones **9a**−**e**
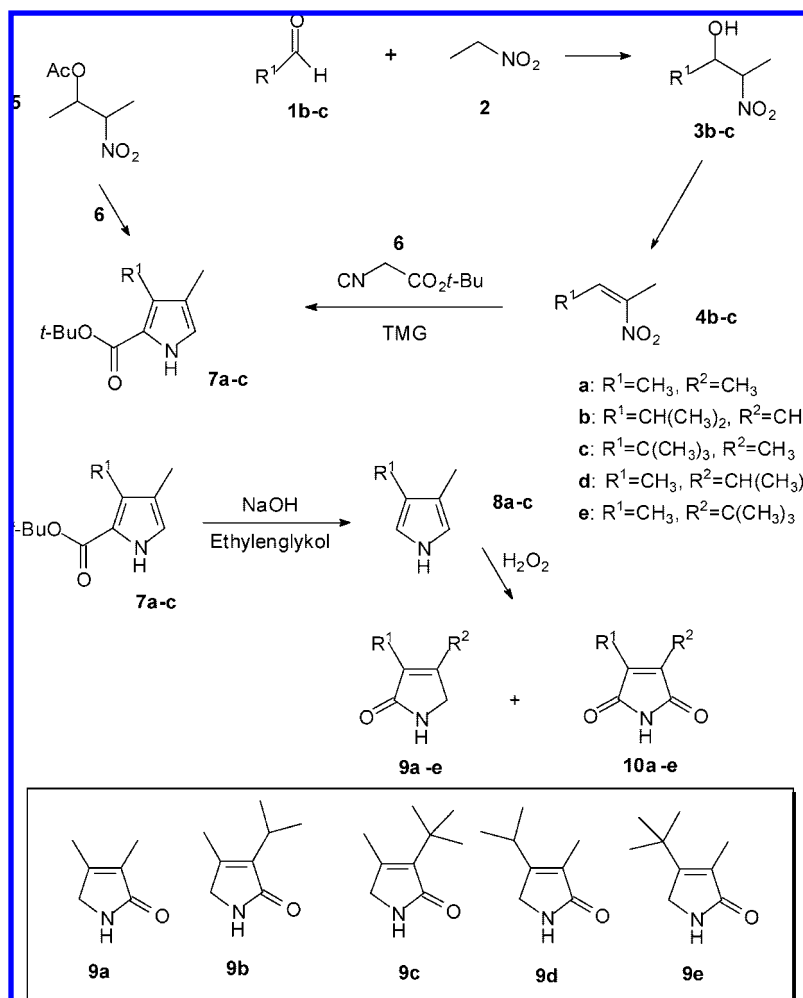
niently usable building blocks (in order to facilitate synthesis and to reduce the overall number of reactions required), and most recently, specifically designed tetrapyrroles have been provided to precisely probe certain biochemical functions of the phytochromes.[15−17] A synthesis of each ring separately was performed here which allows independent access to each position. Following the "convergent" synthesis introduced by Gossauer et al.,[6] the ring-A synthon is coupled with a B-ring derivative by a Wittig reaction, yielding the "left half" of the tetrapyrrole, and a coupling of C- and D-rings provides the "right half" of the target compound. The final condensation of both halves, followed by a saponification of the methyl esters at rings B and C with TFA then yields the tetrapyrrole. If changes are not to be introduced into rings C or D, a cleavage reaction of readily available bilins, e.g., biliverdin, at the central position 10 by utilizing thiobarbituric acid can be used to directly obtain the right half of the tetrapyrrole.[18]

In this study, a series of PCBs was synthesized, which were modified at positions 17 and 18. This aim requires pyrroles with

modified substituents at both positions, 3 and 4, as ring D building blocks. Rings A and B (this one being identical to ring C with respect to its substitution pattern) were synthesized following the routine protocol[6] and were coupled to each other and to the "right" half, following identical procedures in all reactions. It should be mentioned that position 2 at ring A was racemic in all cases.

Taking the non-naturally occurring tetrapyrrole **17a** with a 17,18-dimethyl substitution pattern at positions 17 and 18 as parent compound, two series of substituents at ring D have been synthesized (Figure 1): 17-methyl-18-isopropyl (**17b**), 17-methyl-18-*tert*-butyl (**17c**), 17-ethyl-18-methyl (**17f**), 17-vinyl-18-methyl (**17g**), and 17-isopropyl-18-methyl (**17d**). Including the naturally occurring phytochrome chromophores PΦB (**17h**) and PCB (**17i**), an increase in size of both D-ring substituents was accomplished. Highly substituted pyrroles such as the bilin compounds studied here could be represented with the IUPAC nomenclature but would be unnecessarily complex. Instead we have (see also the Experimental Section) reduced the application of systematic names to cases that have been reported in the literature. Since we feel that phycocyanobilin and biliverdin are commonly used names (see also Figure 1), we prefer to employ these also for the derivatives that we discuss here.
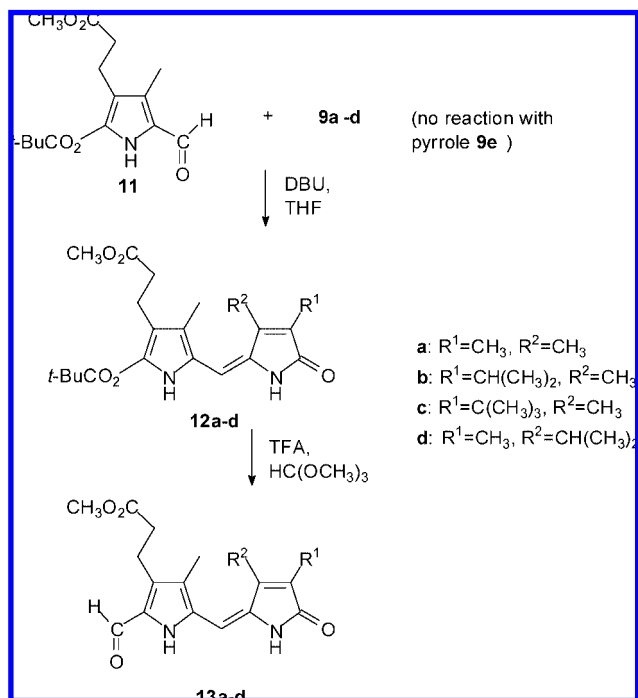
2,3-Substituted pyrroles, the precursors of ring D of tetrapyrroles for this study, were synthesized according to Scheme 1. The introduction of the substituents R[1] and R[2], originating from nitroethane (**2**), was accomplished by using the corre-

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Scheme 2. Synthesis of Formylated Dipyrromethenones **13a−d**

Scheme 3. Synthesis of Novel Open-Chain Tetrapyrroles **17a−g**[a]

[a] The formylation of "right" halves (**13f** and **13g**), obtained from cleavage of biliverdin and mesobiliverdin by thiobarbituric acid, is shown only for **13f**.

sponding aldehydes **1b** and **1c**. Only in the case of $R^1 = R^2 =$ methyl could the commercially available compound (**5**) be used directly. The principle of this synthesis is based on the nonselective oxidation of compounds **8**, leading to a mixture of lactimes **9b** and **9d**, respectively, or **9c** and **9e**, respectively, which, because of different chemical properties (carbonyl position in the vicinity of a methyl or a more bulky isopropyl or *tert*-butyl substituent) could be separated by chromatography. Side products from double oxidation, yielding the cyclic imines **10**, could also be readily removed by chromatography.

The condensation of pyrroles **9a−d** with C-ring building blocks (**11**) was successful in all cases and yielded the corresponding dipyrromethenones **13a−d** (Scheme 2), which could be coupled with the "left half" (**14**) to generate the target tetrapyrroles in the form of their methyl esters (Scheme 3). This final condensation was also performed with **13f** and **13g**, which were obtained from biliverdin and mesobiliverdin after cleavage with thiobarbituric acid. Mesobiliverdin was obtained from biliverdin by reduction of the vinyl groups at positions 3 and 18 with hydrogen. Saponification of the methyl propionates at rings B and C[18] then yielded the modified PCBs/PΦBs **17a−g**, ready for assembly with the apo-phytochrome. Although the overall yield of PCB derivative generation was sufficient, the final amounts of the target compounds were fairly low. We thus refrained from determining the molar extinction coeffients and assume similar values as those determined for the methyl esters.

Whereas the introduction of *tert*-butyl groups at either position, 3 or 4, of **8** could readily be accomplished, leading upon oxidation to compounds **9c** and **9e**, only the 3-*tert*-butyl-substituted compound **9c** allowed condensation with a C-ring synthon yielding the desired dipyrromethenone **13c**. The same reaction using the 4-*tert*-butyl compound **9e** failed, apparently because of steric hindrance between the *tert*-butyl group and the C-ring methyl group.

Assembly of Compounds 17a−g with apo-phy A of Oat. The assembly kinetics and the absorption maxima of the obtained chromoproteins were reported by us in brief.[5] The native
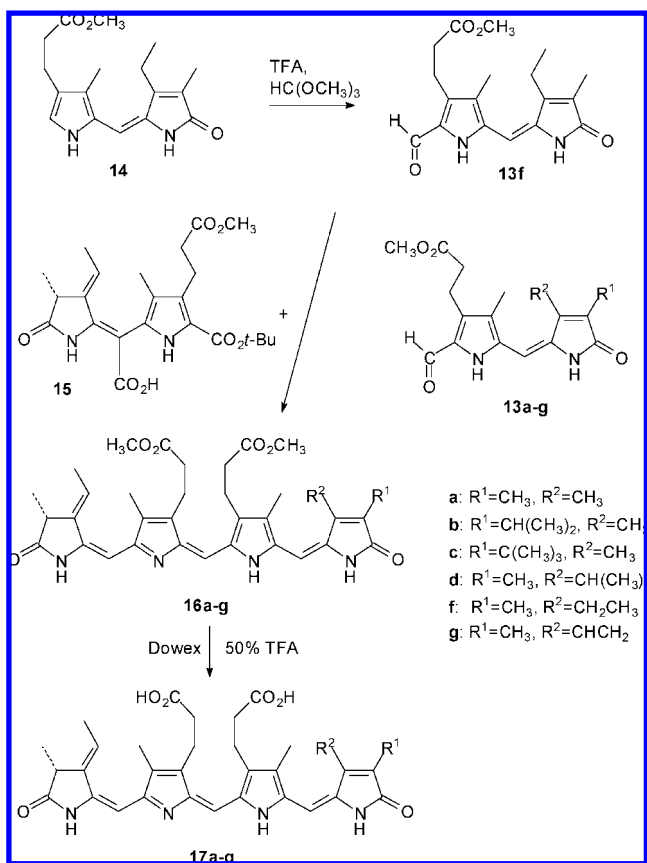
Table 1. Absorbance Maxima of Free Chromophores, Assembled Chromoproteins (P_r, P_{fr}), Assembly Kinetics, and Thermal Stability of Phytochromes Carrying the Chromophores **17a−l**

compound,[a] substitution pattern[b] (pos. 17, 18)	λ_{max} (nm) P_r, P_{fr}	assembly kinetics[c] (min)	thermal stability of P_{fr} (% of P_{fr})[d]
17a, methyl, methyl	655, 714	1.62, 10.8	90
17i, methyl, ethyl	653, 717	0.88, 10.6	92
17h, methyl, vinyl	665, 728	0.38, 2.93	83
17b, methyl, isopropyl	650, 718	6.14, 51.6	96
17c, methyl, *tert* butyl	651, 719	13.2, 58.6	97
17f, ethyl, methyl	658, 707	7.64, 43.8	71*
17 g, vinyl, methyl	663, 714	3.02, 19.6	83
17d, isopropyl, methyl	550, 705	10.5, 156	75

[a] Compounds are listed by increasing substituent size. [b] The naturally occurring chromophores PCB (**17i**), PΦB (**17h**), and their "iso"-forms (**17f** and **17g**) are underlined. [c] The assembly kinetics were fitted in all cases biexponentially. [d] The remaining amount (%) of the P_{fr} form after 48 h is listed, except of compound **17f**; here, the value after 24 h is given (*). All values have been corrected for the percentage of phytochrome which degrades during the long time of incubation, usually in the range of 10%.

chromophore PΦB **17h** gives rise to the most red-shifted absorbance maxima of all chromophores (see Table 1). Already the substitution of its 18-vinyl by an ethyl group (PCB) causes an hypsochromic shift of 10 to 12 nm, which is similar for all other compounds of this group (gathering in the spectral range between 658 and 650 nm for the P_r forms, and between 719 and 705 for P_{fr} forms, respectively). Of outstanding importance is the spectral property of 17-*i*-pr-PCB (**17d**). The chromoprotein assembled with this chromophore shows a remarkable hypso-
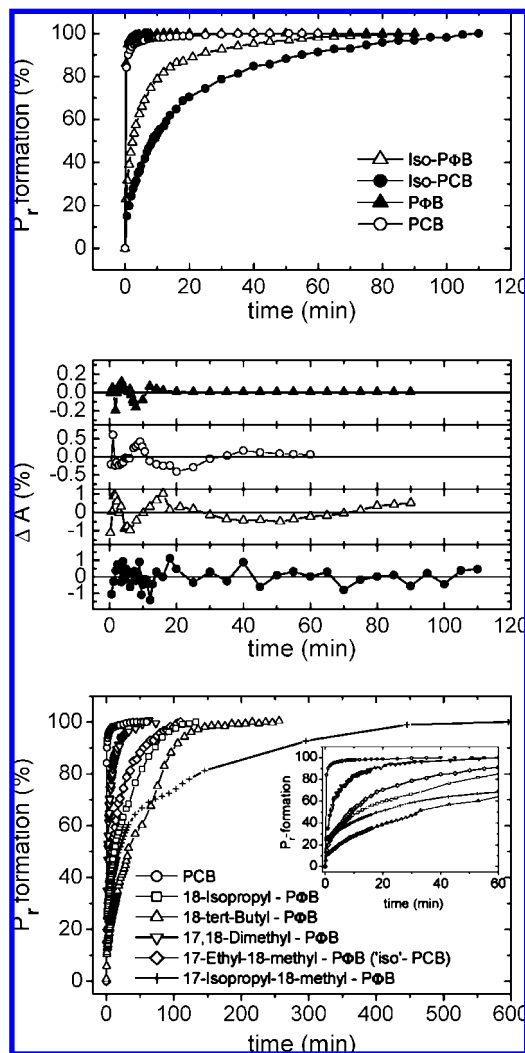
Figure 2. Assembly kinetics of tetrapyrroles **17a−i** with recombinant apophytochrome. Top: Assembly of PΦB and PCB and their iso-forms. Middle: Residuals from biexponential fitting of the assembly traces. Bottom: Assembly kinetics of the remaining bilin derivatives. The assembly kinetics of PCB from top panel is shown again for comparison. Note the different time scales. Inset: Assembly kinetics during the first 60 min.

chromic shift of selectively the P_r band by ca. 100 nm ($\lambda_{max} =$ 550 nm) but not for the P_{fr} band.

The chromoprotein assembly process clearly reveals the optimized interaction of the native chromophore (with respect to its substitution pattern) and the apoprotein, showing a biphasic kinetics with lifetimes of 0.38 and 2.93 min. In all cases, a two-step reaction mechanism could be applied to fit the data. Already the exchange of the 18-vinyl group against the ethyl substituent (PCB) caused a slight retardation of the assembly process (0.88 and 10.6 min, see also Figure 2). It is interesting to note that compound **17a**, carrying at both positions, 17 and 18, the smallest (methyl) substituent does not yield the fastest assembly kinetics (1.62 and 10.8 min). On the other hand, any increase in substituent size causes a strong retardation, most probably due to serious steric interactions with the protein binding site. Here, the change in size at position 17 has a stronger effect (compare PΦB **17h** and its iso-form, **17g**, or PCB **17i** and its iso-form **17f** or the 17-isopropyl-18-methyl compound, Figure 2, top panel).

Thermal Reconversion of the P_r Forms from Light-Induced P_{fr} Forms. Native or recombinantly produced phytochromes are remarkably stable in the dark in both their forms, P_r and P_{fr}.
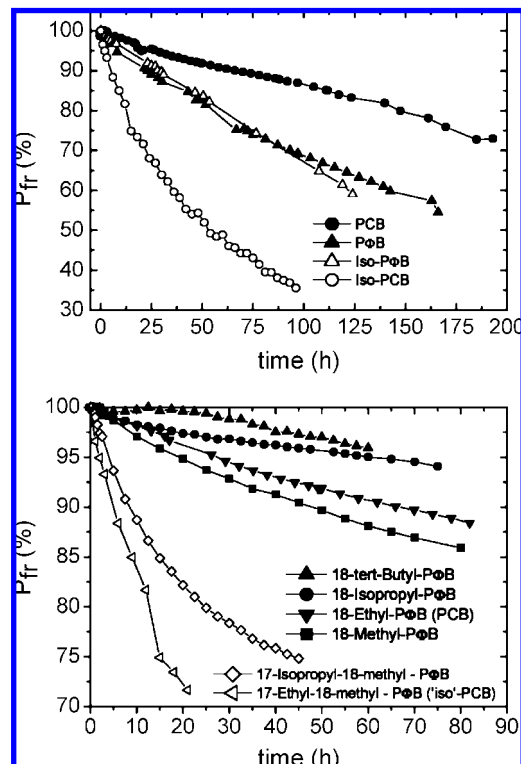

Figure 3. Thermal reconversion of P_r forms from photochemically generated P_{fr} for phytochromes carrying tetrapyrroles **17a−i** as chromophores. Top: PΦB and PCB, and their iso-forms, respectively. Bottom: Remaining bilin derivatives. Note the different time scales.

Whereas the P_r forms show no thermal conversion into the P_{fr} forms at all, the P_{fr} forms convert slowly in the dark by thermal activation into the P_r forms (Figure 3). Since during long observation times a degradation of the protein also occurs (which has been monitored and used for correction of the experimental results), no exponential fit could be employed. Thus, the remaining amount of P_{fr} after two days of observation is given for comparison (Table 1). In this series of novel chromophores, the thermally most stable chromoprotein reconverted back into the P_r form by less than 3% within 48 h at 20 °C (18-*tert* butyl, compound **17c**). In the thermal conversion process, those compounds with the most bulky substituents at position 18 showed the highest stability (18-isopropyl and 18-*tert* butyl). These chromoproteins were even more stable than those carrying the native chromophores PΦB or PCB. The most unstable phytochromes carried chromophores with increased size of substituent 17, i.e. 17-ethyl and 17-isopropyl, which were converted to P_r by ca. 20 and 30%, respectively, already after an incubation for one day (24 h). Also for these latter two chromoproteins, no significant degradation took place (loss by degradation was below 10% over the observation time period), but the loss of P_{fr} absorbance was completely due to P_r reformation.

Time-Resolved Absorbance Changes during the Light-Induced P_r to P_{fr} Conversion (laser-flash photolysis). Following the ultrafast ps primary photochemistry,[19] plant phytochromes form an intermediate which can be trapped at low temperature and which shows a bathochromic shift of its absorption maximum by ca. 30 nm ("I_{700}").[8] This species already carries the chromophore

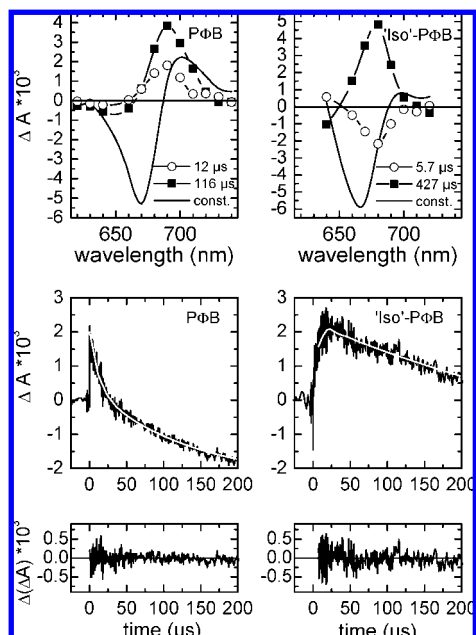(19) Müller, M. G.;Lindner, I.;Martin, I.;Gärtner, W.;Holzwarth, A. R. *Biophys. J.* **2008**, *94*, 4370–4382.

Figure 4. Top: global fit for I_{700} formation and decay of PΦB- and "iso"-PΦB-containing chromoproteins. Middle: Single wavelength trace (680 nm) from 0 to 200 μs. Bottom: Fitting residuals for the 680 nm traces.

in its photoisomerized form (5-Z,10-Z,15-E).[11,20] The following, thermally driven processes yield one or more intermediates with a low extinction coefficient ("I_{bl}", bl = bleach) which form within several microseconds and eventually convert into a P_{fr}-like and finally into the stable P_{fr} form. The complete formation of P_{fr} can last several seconds. As part of this photoconversion of phytochrome, it is discussed that several isoforms of P_r might be present, and that also equilibria between several intermediates exist.[21] An analysis of these microsecond-to-second processes is only possible by a global fit procedure, which yields the lifetimes of the various processes and also the spectra of the intermediates related to these kinetic processes. It has to be kept in mind that the spectra shown here are difference spectra which are mixed with the contributions of other intermediates, present at that time point of observation. By convention, intermediates that decay are shown with positive amplitudes, whereas intermediates that are formed with a certain lifetime are presented with negative amplitudes.

Obvious effects of substituent variation are shown in Figures 4 and 5, comparing the early processes (up to one ms) of phytochromes carrying PΦB (**17h**), "iso-PΦB (**17g**), PCB (**17i**), and "iso"-PCB (**17f**). Whereas native phytochrome shows two decay kinetics of ca. 10 and 100 μs for the I_{700} intermediate (Figure 4 and Table 2), its iso-form shows only one decay process (427 μs). For this derivative, an additional process is detected in the short time range, which, however, reflects a rise process with a lifetime of 5.7 μs. Here, the change in the substitution pattern causes a slowing of both the formation and the decay kinetics of the I_{700} intermediate. This is even clearer in the single wavelength traces (680 nm, Figure 4, lower panel), where the biexponential decay of native phytochrome and the rise and the decay of "iso"-PΦB-phytochrome are shown.

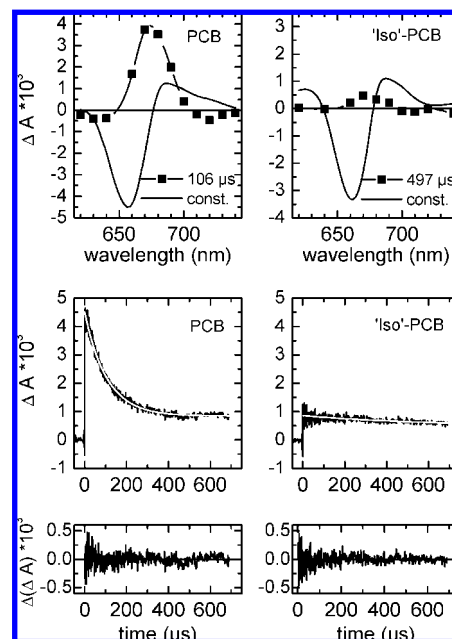An even stronger deviation of the normally observed absorbance changes was found for "iso"-PCB (Figure 5). Again, the

Figure 5. Global fit analysis for I_{700} formation of PCB- and "iso"-PCB-containing chromoproteins. Middle: Single wavelength trace (680 nm) from 0 to 200 μs. Bottom: Fitting residuals for the 680 nm traces.

"iso"-form is compared to the naturally occurring chromophore PCB.[22,23] PCB shows a kinetic behavior similar to the native chromophore PΦB, except that the I_{700} decay is monoexponential with a time constant of 106 μs (see also ref 24). Also here, the "iso"-form decays much slower (ca. 500 μs), but more importantly, this intermediate shows a strongly diminished extinction coefficient, as also seen in the single wavelength trace (680 nm, lower panel of Figure 5). This reduced absorbance of the I_{700} intermediate is not due to a lower overall conversion, i.e., a reduced quantum yield, as can be seen in Figure 6. Again in comparison to PCB-phytochrome, the longer time scale shows the P_{fr} formation. For both chromoproteins, two dominating processes for the P_{fr} formation can be identified: 2.8 and 25 ms for PCB, and 12 and 328 ms for "iso"-PCB. Although slower in the P_{fr} formation, the amount of P_r that is converted into P_{fr} is similarly large in both chromoproteins (see curve without symbols, representing the "constant" at the end of the experiment, which corresponds to the final P_{fr} − P_r difference spectrum).

A change in kinetics is found also for the dimethyl derivative, in particular for the formation of the P_{fr} form (Table 2, Figure 7). Whereas the I_{700} intermediate decays very similar to the PCB-chromoprotein with monoexponential kinetics ($\tau = 116$ μs), the P_{fr} formation is different from the PCB-containing phytochrome. The dominant P_{fr}-forming process of the PCB-protein ($\tau = 25.4$ ms) with a positive (around 640 nm) and a negative branch (around 720 nm) is also found for the dimethyl derivative (640/720 nm, Figure 7) yet with a slightly changed lifetime ($\tau = 37.5$ ms). The other important P_{fr}-forming component in the case of the PCB-assembled phytochrome is a faster process of bilobal shape and with a time constant of 2.8 ms (open circles in Figure 6). This process has no equivalent in the protein containing the dimethyl compound: although the global fit yields

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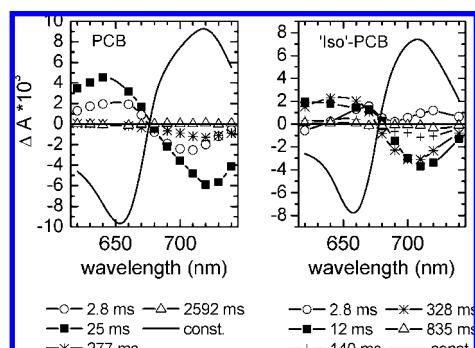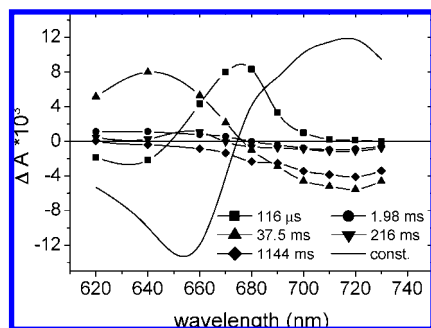
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Table 2. Lifetimes of Time-Resolved P_r-to-P_{fr} Conversion[a]

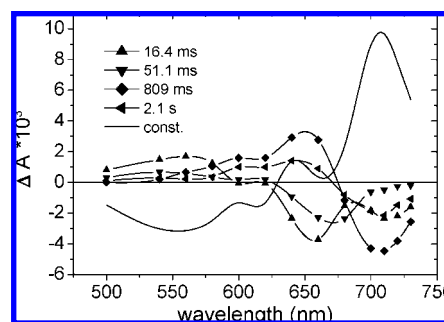| compound, substitution pattern[b] (pos. 17, 18)	$\tau_0{}^c$ (μs)	τ_1 (μs)	τ_2 (μs)	τ_3 (ms)	τ_4 (ms)	τ_5 (ms)	$\tau_6{}^d$ (s)
17a, methyl, methyl	−	−	116	1.98	37.5	216	1.14
17i, methyl, ethyl	−	−	106	2.8	25.4	277	2.59
17h, methyl, vinyl	−	12	116	3.3	41	152	1.2
17b, methyl, isopropyl	4.75	−	243	2.5	16.8	51.8	137
17c, methyl, *tert* butyl	−	37	125	2.45	18	65	62
17f, ethyl, methyl	−	−	497	2.8	12	140/328[e]	0.83
17 g, vinyl, methyl	5.7	−	427	−	25/75[e]	237	1.86
17d, isopropyl, methyl	−	−	93/243[e]	−	16.4/51.1[e]	809/2100[e]	−

[a] Compounds are listed by increasing substituent size. τ_1 and τ_2 refer to the decay of the I_{700} intermediate, whereas τ_3 to τ_6 are related to a rise of the P_{fr} form. [b] The naturally occurring chromophores PCB (**17i**), PμB (**17h**), and their "iso"-forms (**17f** and **17g**) are underlined. [c] The time constant τ_0 has been included in order to identify I_{700} formation kinetics. [d] This very long time constant is always of very low intensity and is required only for a sufficient fit. [e] According to the similar shape of the kinetic traces, these time constants are assumed to represent the same reaction process. In some cases, the fitting procedure splits such process into two separate kinetics, depending on the quality of the recorded data.

Figure 6. Time-resolved P_{fr} formation of (left) PCB- and (right) "iso"-PCB-containing phytochromes.

Figure 7. Global fit analysis for 17,18-dimethyl PCB-containing phytochrome.

Figure 8. Time-resolved P_{fr} formation of the 17-isopropyl-18-methyl chromophore-containing phytochrome.

This chromoprotein is apparently present as a mixture of conformations in the P_r state. The initial processes (below 1 ms) are thus not clearly determined, yielding two time constants of ca. 90 and 240 μs. The slower processes (P_{fr} formation) confirm this strongly disturbed interaction between chromophore and protein, making an unambiguous assignment very difficult. The earliest process in the millisecond time domain (Figure 8) is a decay of a broad, unstructured absorbance peaking around 550 nm. This form significantly resembles the P_r absorption of this derivative. This decay leads to the formation of probably two intermediates (negative maxima around 660 and 710 nm). The second process (51.1 ms) describes again the formation of an intermediate absorbing around 675 nm. The P_{fr} form is generated by two processes with similar overall contours, yet different intensities, i.e., the 809 ms and 2.1 s processes. Both reactions show a decay around 650 nm, concomitant with a rise around 710 nm (P_{fr} form). The "constant" curve, showing the residual absorbance at the end of the experiment is identical to the independently determined steady-state difference spectrum $P_{fr} - P_r$ (see for comparison ref 5).

3. Discussion

We have presented a synthetic approach toward linear tetrapyrroles, which allows a high degree of substituent variation. The convergent synthesis, i.e., the generation of each ring separately, followed by condensing two pyrrole components to form the "right" and two pyrrole components to form the "left" half of the molecule, and finally condensing both pyrromethenones to the full length tetrapyrrole, allows maximal variability of the substitution pattern of each ring. The very mild hydrolysis of the esterified propionic acid side chains in the last step yields tetrapyrroles with biological relevance in satisfactory amounts. This approach can also be applied for the

a 2 ms component, this process has practically no absorption intensity (filled circles in Figure 7). Instead, the second important contribution to P_{fr} formation in the case of the dimethyl derivative is a much slower process of 1144 ms, which reflects only a P_{fr} rise around 720 nm but no decay in the short wavelength region.

Also the 18-isopropyl- and the 18-*tert*-butyl substituents cause changes in the photochemical behavior: a rise of the I_{700} intermediate with a maximal absorbance around 680−690 nm is detected for the 18-isopropyl compound (4.75 μs) followed by its decay with a lifetime of ca. 240 μs, whereas the 18-*tert*-butyl compound shows two decay processes of 37 and 125 μs for its I_{700} intermediate. The formation of P_{fr} (18-isopropyl compound) takes place with three lifetimes of 2.5, 16.8, and 51.8. ms. The P_{fr} formation of the 18-*tert*-butyl derivative is very similar to the 18-isopropyl compound, also giving three rise components of 2.45, 18, and 65 ms (Table 2).

The strongest changes on the time-resolved P_r to P_{fr} conversion was found for the 17-isopropyl-18-methyl chromophore.

generation of tetrapyrroles carrying stable isotopes (^{2}H, ^{15}N or ^{13}C) at selected positions.[25]

The failure of generating a 17-*tert*-butyl-substituted PCB indicates the steric constraints within the tetrapyrrole structure. A *tert*-butyl group would strongly interfere with the methyl substituent of the adjacent C-ring and would induce a twisting around the connecting methine bridge and a strong reduction of the π-electron conjugative system.

Absorption Maxima of Assembled Chromoproteins. The effect of D-ring substituent modification has recently been reported for some of the compounds presented here.[5] Whereas the reduction in size of the substituents at position 17 (dimethyl derivative) has no destabilizing effect for either the thermal stability of the protein toward degradation or the stability of the P_{fr} form, any increase in size causes effects on the positions of the P_r and P_{fr} absorption maxima, which are blue-shifted up to ca. 10 nm. This can be rationalized because of an incremental increase in substituent size, causing a growing disturbance of the chromophore−protein interactions. However, no monotonous change can be expected (cf. 18-isopropyl and 18-*tert*-butyl), since the binding site of the protein has to be assumed to provide limited space and flexibility. An exceptionally different situation is caused by the 17-isopropyl-18-methyl derivative which selectively in the P_r form shows a 100 nm hypsochromic shift, whereas the P_{fr} absorption maximum falls into the same range as the other modified PCBs. The absorbance of this chromoprotein resembles that of phycoerythrobilin-assembled phytochrome,[26] which has a conjugated π-system extending only over the A-, B-, and C-rings, because of its 15,16 single bond. Since the free chromophore absorbs in the same wavelength range as other tetrapyrroles ($\lambda_{max} = 365$, 587, and 631 nm; see also Experimental Section), this hypsochromic shift is not an inherent property of that compound but is induced by the protein environment. Apparently, the protein cavity does not tolerate a large substituent at that position (17) in the P_r form and forces the D-ring into a conformation which does not allow extended conjugation with the other three rings. The situation is different in the P_{fr} state where apparently sufficient space is available within the binding pocket to accommodate the large isopropyl substituent.

Assembly Kinetics. An increase in size of the substituents at positions 17 or 18 causes a slowing-down of the assembly process, as might be assumed because of the increased steric interactions of the tetrapyrrole compound with close-by amino acids. This behavior is seen for both series, yet changes at position 17 yield a stronger effect, with the slowest assembly kinetics for the 17-isopropyl-18-methyl derivative with a half-life of 156 min for τ_2. This extremely slow process, most probably due to specific, strongly hindering interaction at that particular position of the protein, is also in accordance with the strongly blue-shifted absorption maximum of that compound. It is of interest to note that it is not the smallest substituent (methyl group) that leads to the fastest assembly. Instead, the vinyl group of the native chromophore yields the fastest τ_1 and τ_2, followed by PCB, carrying an ethyl group. The vinyl group is advantageous over the ethyl group, even if it is placed at the "wrong" position (pos. 17). Again, "iso"-PΦB assembles faster

than "iso"-PCB, indicating that a substituent with π-electrons supports the interaction with the protein possibly by an interaction with an aromatic amino acid (Table 1).

Thermal Stability of the P_{fr} Form. The increased size of the substituent at position 18 stabilizes the P_{fr} form in the thermal P_r-reformation process. The two compounds 18-isopropyl- and 18-*tert*-butyl-PCB yield more stable chromoproteins than those of native or PCB-assembled phytochrome. The noticeable regeneration of P_r might be a property of phytochrome that has developed in order to provide a sufficient amount of the P_r form under dark conditions; alternatively, the observed thermal P_r generation allows a slow deactivation of the physiologically active P_{fr} state even in the dark.

Time-Resolved P_r to P_{fr} Conversion. The flash photolysis studies revealed important interactions between chromophore and protein that occur during the dynamics of the chromoprotein photoconversion. Since the time resolution of the system is limited to several hundred nanoseconds, the formation of the I_{700} intermediate of the native system is "instantaneous", i.e., the first observable event is the decay of this species with time constants of ca. 10 and 100 μs, respectively,[24] (for earlier processes see ref 19). The smaller substituent at position 18 does not significantly change the kinetics of the I_{700} decay or of the P_{fr} formation. The I_{700} intermediates decay with ca. 110 μs for the PCB chromophore and with a nearly identical time constant for the dimethyl chromophore. It is also a millisecond process in both chromoproteins that contributes mostly to the P_{fr} formation (25 ms for PCB and 37 ms for the dimethyl compound). However, whereas in the PCB protein this 25 ms-process is maximally contributing to the P_{fr} formation, in the dimethyl derivative there is a second, much longer lasting process of more than 1 s that is similarly intense during P_{fr} formation. One can propose that the longer times during the P_{fr}-forming reactions can be ascribed to an accommodation of the chromophore within the binding pocket, where the overall conformation has already been adapted. In a process like that, the smaller methyl group at position 18 would be less effective to fix the D-ring in its final position. The chromoproteins carrying chromophores with larger substituents at position 18 (iso-propyl and *tert*-butyl) still show an I_{700} decay in the hundreds μs range but, interestingly, also show faster components in the P_{fr} formation than the native system: in native phytochrome, the dominant component for P_{fr} formation is a 40 ms process (25 ms for PCB); in the 18-isopropyl compound an ensemble of 2.5, 16.8, and 51.8 ms processes contribute equally to the P_{fr} formation, similar to that for the 18-*tert*-butyl chromophore, where a 2.45 and an 18 ms process contribute equally to the P_{fr} formation.

Interestingly, the vinyl substituent at position 17 significantly slows the I_{700} formation such that it becomes observable in the ns−μs detection range. For "iso"-PΦB-phytochrome, a rise component around 700 nm could be observed with $\tau = 5.7$ μs. The size-increased substituent at position 17 (ethyl, vinyl, and isopropyl) in all three cases leads to a slowed-down P_{fr} formation, which in the last cases (isopropyl) extends into the seconds range, clearly an effect of a strongly disturbed interaction between the chromophore and its protein surrounding.

4. Experimental Section

Biochemical Preparations. The N-terminal domain of phytochrome A from oat (*Avena sativa*), spanning amino acids 1−596, was produced by heterologous expression in the methylotrophic

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yeast *Hansenula polymorpha* following published procedures.[27] The cDNA was furnished at its 3′-end by an octadecameric sequence encoding for a His$_6$-tail at the C-terminal end of the recombinant protein that allows facile affinity purification. Assembly with PCB or synthesized novel chromophores was routinely performed in the crude cell lysate, except for cases where assembly kinetics were determined. After assembly, the samples were further treated in dim or in safe green light. Affinity-purified chromoproteins were relieved from imidazole (used as eluting agent up to a concentration of 200 mM) and adjusted to the required buffer and protein concentration.

Assembly Kinetics. A buffered solution (pH 7.5) of affinity-purified apo-phytochrome (1 mL, ca. 5 μM) in a quartz cuvette was placed into a spectrophotometer, and 2−4 μL of a concentrated solution (ca. 5 mM in DMSO) of the various chromphores were rapidly added. After a short mixing, the rise of the P$_r$ absorption was followed at the maximum of the absorption band. Routinely, the first data point could be recorded after ca. 15 s.

Thermal Formation of P$_r$ Form from Photochemically Generated P$_{fr}$. Buffered solutions (pH 7.5) of fully assembled chromoproteins were maximally converted into the P$_{fr}$ forms by saturating illumination under temperature-controlled conditions (20 $\pm$ 1 °C), and an absorption spectrum was recorded (= 100% P$_{fr}$). The sample was then kept in the spectrophotometer in the dark, and the absorbance at the P$_{fr}$ maximum was read at given time points. At the end of the measurements, again the amount of the P$_{fr}$ form after saturating illumination was determined. Because of degradation of the samples, this value for P$_{fr}$ was always smaller than at the begin of the experiment (routinely by ca. 10%). The data for the thermal conversion process were then corrected for the degree of degradation.

Steady-State and Time-Resolved Absorption Spectroscopy. For all spectroscopic measurements, the sample concentration was in a similar range of A = 0.2−0.3 (1 cm path length). For conversion experiments between the P$_r$ and the P$_{fr}$ state, samples were initially treated with light >715 nm (RG9 cutoff filter), until no further increase in absorbance of the P$_r$ form was observed. Then, irradiation through interference filters of appropriate wavelengths was performed for several minutes, followed by the recording of absorbance spectra, until maximal formation of the P$_{fr}$ forms was found. The reformation of the P$_r$ state from P$_{fr}$ was performed in a similar time-controlled manner using an RG9 cutoff filter.

Time-resolved absorption spectroscopy and the analysis of the kinetic data were performed utilizing the same dual-beam setup and global fit program as formerly described.[28] The analogue phytochromes were always excited by the laser beam at their absorption maxima. (i) Special care was given to achieve nearly identical concentration (absorbance amplitude) of the samples, and (ii) an energy dependence of the signal intensity was always performed before the measurement series in order to avoid nonlinear photoprocesses and on the other hand to avoid the build-up of a light gradient within the cuvette (for further details see ref 28).

Chemical Syntheses. Anhydrous solvents were obtained by literature procedures.[29] Reactions were conducted under argon when anhydrous solvents were used. ^{1}H and ^{13}C NMR spectra: Bruker DRX-500, AM-400, DRX-400, AC-270, ARX-250; NMR signals are given in δ (ppm) relative to traces of undeuterated solvents, and coupling constants J are given in Hertz. UV−vis spectra: Shimadzu UV-2102 PC and UV-2401 PC; λ_{max} (ϵ) in nm. MS in *m/z*. Static pressure column chromatography (CC): Merck silica gel 60 (40−63 μm) was routinely used. Preparative thin layer chromatography (TLC): Merck silica gel 60, 2 mm. Ion exchange resin: Dowex 50WX8−200 (Aldrich). Abbrevations: DBU = 1,8-diazabicyclo-[5.4.0]-undec-7-ene; DCC = dicyclohexylcarbodiimide; DMAP = dimethylaminopyridine; TFA = trifluoroacetic acid; TMG = *N,N,N′,N′*-tetramethylguanidine.

The reaction products in all cases could be isolated as pure compounds, which were unambiguously characterized by routine spectroscopic methods. For a detailed description of the reactions, the purification protocols, the obtained yields and the spectroscopic identification, please see the Supporting Information.

5. Conclusion

Chemically synthesized phytochrome chromophores with incrementally increasing size of the substituents at the photo-isomerizing D-ring demonstrate the subtle interactions between chromophore and protein also for dynamic processes. The binding site of apo-phytochrome has been optimized during the evolution of the protein for the native chromophore and does not tolerate larger substituents at the "inner" position 17. Also, an increase in size a the "outer" position 18 interferes with the function of phytochrome, as well in the dark-recovery kinetics of P$_r$ from P$_{fr}$, as also in the light-induced formation of the P$_{fr}$ state. Interestingly, exactly a C$_2$ substituent (vinyl or ethyl) is required for an efficient fixation of the D-ring during the P$_{fr}$ formation (see the slowed process of the dimethyl chromophore). The application of structurally modified chromophores demonstrates how precise the protein binding pocket tunes the reactivity of the chromophore in order to guarantee the outstanding spectral and kinetic properties of this plant photoreceptor.

Supporting Information Available: Detailed description of the reactions, the purification protocols, the obtained yields, and the spectroscopic identification. This material is available free of charge via the Internet at http://pubs.acs.org.

JA076728Y

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