Regioselective Deuteration and Resonance Raman Spectroscopic Characterization of Biliverdin and Phycocyanobilin**

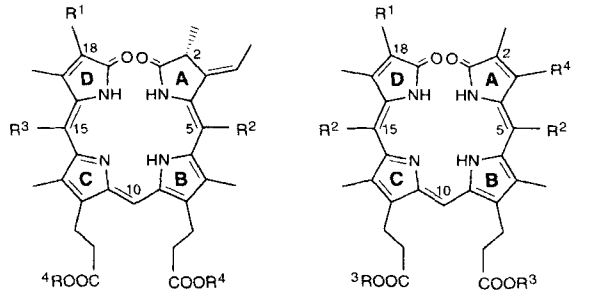
Bernd Knipp, Krista Kneip, Jörg Matysik, Wolfgang Gärtner,* Peter Hildebrandt,* Silvia E. Braslavsky, and Kurt Schaffner

Abstract: 5-Deuteriophycocyanobilin (**4**), 5,15,18^2,18^2-tetradeuteriobiliverdin IXα dimethyl ester (**6**), and 5,15-dideuteriomesobiliverdin IXα dimethyl ester (**8**) have been prepared and analyzed by resonance Raman spectroscopy in the C–H out-of-plane region, based on a previous normal-mode analysis of biliverdin XIα dimethyl ester (**5**). In this way most of the modes containing out-of-plane vibrations of the methine bridges could be assigned unambiguously. These results are of special relevance with regard to the interpretation of the resonance Raman spectra of the chromoprotein phytochrome.

Keywords
isotopic labeling · phytochrome · Raman spectroscopy · tetrapyrroles · vibrational analysis

Introduction

Open-chain tetrapyrroles such as phytochromobilin (**1**)[1] or phycocyanobilin (**2**)[2] serve as prosthetic groups in certain biological photoreceptors (Figure 1).[3] Both compounds originate biosynthetically from biliverdin IXα (**3**), formally by a diene 1,4-saturation of ring A and, in the case of **2**, by further saturation of the vinyl group of ring D.[4] In the chromoproteins both compounds are covalently attached to a cysteine residue through a thioether bond. In this way, **2** is the visible light absorbing chromophore of C-phycocyanin, one of the chromoprotein constituents of the light-harvesting antennae (phycobilisomes) of some algal photosynthetic systems. Its conformation, which has been fully determined by X-ray analysis,[5] remains unchanged during the functional lifetime of the chromophore. The situation is different for **1** in phytochromes, which mediate photomorphogenesis in plants by photochromic interconversion of the two thermally stable forms, the red absorbing P_r and the far-red absorbing form (P_{fr}).[3, 6] The primary photoreactions associated with the chromophore in the light-induced $P_r \rightleftharpoons P_{fr}$ interconversions have been recognized by NMR[6a, 7] and, more recently, by resonance Raman (RR) spectroscopy[8, 9] of oat phytochrome A as (*E,Z*) isomerizations around the C-15,16 double bond. These photoprocesses are followed by cascades of thermal conformational equilibrations of the chromophore and the surrounding protein, the structural aspects of which have not yet been resolved in detail.[10] One of the difficulties that have been encountered in the molecular elucidation of these events lies in the fact that the extraction of detailed structural information encoded in the RR spectra requires reliable—hitherto unavailable—vibrational assignments for linear tetrapyrroles in solution and, ultimately, in the protein. As an approach to partly unravel the complexity of tetrapyrrole Raman spectra, we describe here the preparation of regioselectively deuterated biliverdins and phycocyanobilins and the analysis of their RR spectra.



	R^1	R^2	R^3	R^4
1	$CH=CH_2$	H	H	H
2	CH_2CH_3	H	H	H
4	CH_2CH_3	D	H	H
9	CH_2CH_3	H	H	CH_3

	R^1	R^2	R^3	R^4
3	$CH=CH_2$	H	H	$CH=CH_2$
5	$CH=CH_2$	H	CH_3	$CH=CH_2$
6	$CH=CD_2$	D	CH_3	$CH=CH_2$
7	CH_2CH_3	H	CH_3	CH_2CH_3
8	CH_2CH_3	D	CH_3	CH_2CH_3

Figure 1. Structural formulas of open-chain tetrapyrroles. Left: phycocyanobilin- and phytochromobilin-derived structures; right: biliverdin-derived structures.

[*] W. Gärtner, P. Hildebrandt, B. Knipp, K. Kneip, J. Matysik, S. E. Braslavsky, K. Schaffner
Max-Planck-Institut für Strahlenchemie, Postfach 101365
D-45413 Mülheim an der Ruhr (Germany)
Fax: Int. code +(208)306-3951
e-mail: hildebrandtp@mpi-muelheim.mpg.de
gaertner@dsa. mpi-muelheim.mpg.de

[**] **Abbreviations:** A - B, B-C, C-D, dipyrrole units of tetrapyrrole compounds; e.g., A-B, rings A and B. FT IR, Fourier-transform infrared. HOOP, hydrogen-out-of-plane. PED, potential energy distribution. RR, resonance Raman.

Only a few approaches to regioselective H/D exchanges in bilatrienes have been reported.[11, 12] When the symmetrical octaethylbiliverdin, which represents a model verdin system, is subjected to electrophilic substitution in the presence of CF_3CO_2D at 100 °C, H/D exchange occurs preferentially at C-5 and C-15 of the lateral methine bridges. For phycocyanobilin (**2**) deuteration with CH_3OD has been reported to occur at positions C-2, C-3, and C-15 (assignment by 100 MHz ^{1}H NMR).[12] However, the labeled product had not been isolated and purified for a sufficiently reliable analysis. Since **2** is more readily accessible (from algae) than phytochromobilin (**1**) and therefore has often been used as a substitute for the natural chromophore in phytochrome reconstitution experiments, deuterated isotopomers of **2** are of special interest in this respect. Moreover, the photophysical and photochemical properties of wild-type phytochrome are quite similar to those of the recombinant chromoprotein containing phycocyanobilin.[13] An RR study of a recombinant, phycocyanobilin-reconstituted phytochrome should therefore afford valuable general information about the structural changes of chromophore and the protein surroundings involved in the $P_r \rightleftharpoons P_{fr}$ conversion.

In the following we report on the synthesis of 5-deuteriophycocyanobilin (**4**), 5,15,18^2,18^2-tetradeuteriobiliverdin IXα dimethyl ester (**6**), and 5,15-dideuteriomesobiliverdin IXα dimethyl ester (**8**), as well as the RR spectroscopic analysis of these compounds.

Results and Discussion

Synthesis of the Tetrapyrrole Isotopomers: Treatment of biliverdin IXα dimethyl ester (**5**)[14] with CF_3CO_2D in a sealed NMR tube at 100 °C, followed by re-esterification yielded its (5,15,18^2,18^2) tetradeuterated isotopomer (**6**) with 85 atom% D incorporation in an overall yield of 61%. Experimental attempts to increase the extent of deuterium incorporation by prolonging the reaction time led to a remarkable decomposition of the starting material. The regioselectivity of the deuteration is in accordance with ab initio calculations for an electrophilic attack on **5**.[15] A D/H back-exchange in **6**, which is possible based on a priori considerations, was not observed when a solution of the isotopomer in chloroform/1% methanol was shaken with 0.1 M hydrochloric acid.

In order to restrict the H/D exchange to the C-5 and C-15 positions, mesobiliverdin IXα dimethyl ester (**7**)—carrying ethyl rather than vinyl groups at C-3 and C-18—was subjected to CF_3CO_2D at 100 °C, yielding the 5,15-dideuterio product **8** (>97 atom% D) in 97% yield.

When phycocyanobilin (**2**) was treated with CF_3CO_2D at ambient temperature, ^{1}H NMR monitoring showed that the highest-field methine bridge proton was replaced (Figure 2). Based on NOE data[16] this can be attributed unequivocally to deuterium incorporation at C-5, that is, formation of **4** (92 atom% D in 93% yield), in contrast to a previous assignment in favor of D incorporation at C-15.[12] Our interpretation is also in accord with a deuteration of phycoerythrobilin,[17] in which deuterium was introduced at C-5, at a position chemically comparable to C-5 of **2**. An H/D exchange at the asymmetric carbon C-2 of **2**, which is known to occur in CH_3OD[12] and

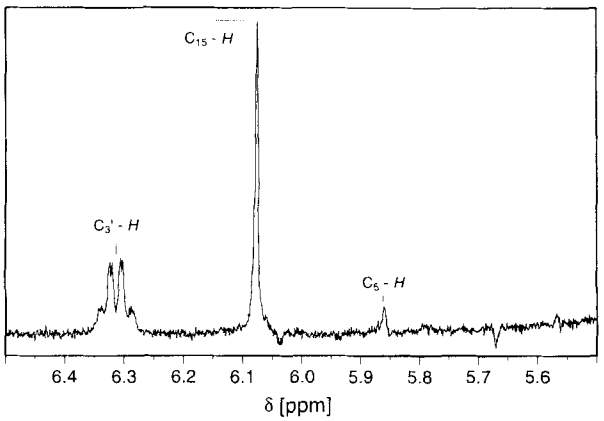


Figure 2. ^{1}H NMR spectrum of **4** in the region δ = 5.5–6.5.

simultaneously to cause racemization,[18] was not observed in CF_3CO_2D. D/H back-exchange at C-5 was achieved simply by dissolving **4** in chloroform containing 1% methanol and shaking with aqueous acetic acid. For RR spectroscopic measurements **2** and **4** were converted into the potassium salts by treatment with acetonitrile/potassium phosphate buffer.

Since the native chromophore of phytochrome, phytochromobilin (**1**), is available by methanolysis of algae,[1] we wish to note that, in analogy to the preparation of **6** from **5** (vide supra), a preparative approach to the synthesis of 5,15,18^2,18^2-tetradeuteriophytochromobilin becomes possible. Further, if the selective D/H back-exchange at position 5, reported for **4**, could also be accomplished for tetradeuterated phytochromobilin, its 15,18^2,18^2-trideuterio isotopomer would become available. In particular the synthesis of this latter compound is of major importance since all deuterium labels are located in ring D, which undergoes conformational changes during the phototransformation of phytochrome.

Resonance Raman Spectroscopic Characterization: For biliverdin dimethyl ester IXα (**5**), a normal-mode analysis has been carried out previously by using a semiempirical (AM1) force field[19] scaled according to Pulay's method.[20] Based on the calculated frequencies as well as IR and RR intensities, plausible assignments of the observed vibrational bands had been suggested. These assignments were found to agree with the IR and RR spectra of a sample of **5** deuterated at the pyrrole nitrogen atoms.[19]

The comparison of the RR spectrum of **5** with those of **7** and phycocyanobilin dimethyl ester (**9**) reveals distinct spectral differences particularly in the frequency regions between 1100 and 1500 cm^{-1} (Figures 3 and 4). This can readily be rationalized in terms of the compositions of the normal modes, which, owing to the significant contributions of the ring side chains, should in fact sensitively reflect the different substitution patterns of these compounds. Since C–H in-plane bending vibrations of the methine bridges are also involved in these modes, it is not surprising that this spectral region displays distinct differences upon deuteration of the vinyl substituents and/or the methine bridges in **6** and **8** (Figure 3). Similar considerations also hold for **2** and its deuterated isotopomer **4** (Figure 4) although the single D/H exchange at C-5 causes less spectral changes as compared to the

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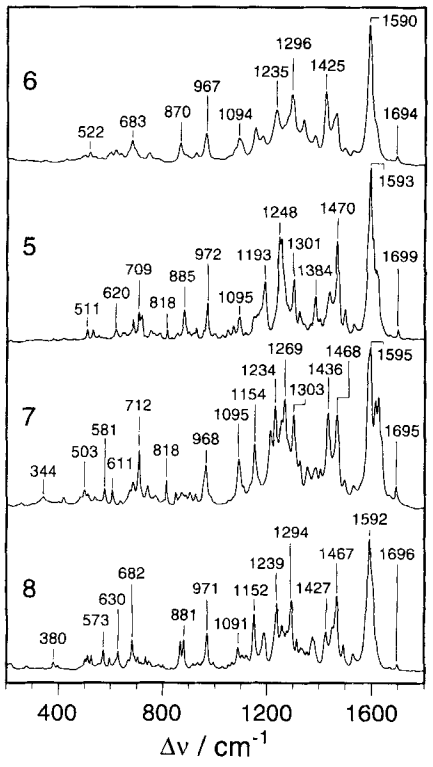

Figure 3. RR spectra of **5**, **6**, **7**, and **8** between 200 and 1800 cm^{-1}.

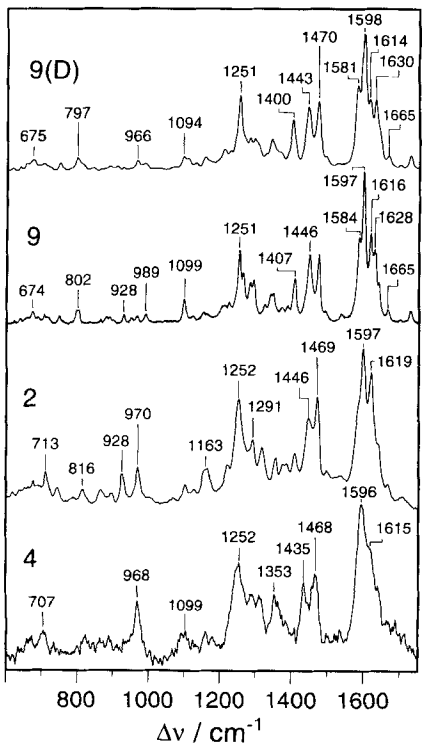

Figure 4. RR spectra of **2**, **4**, **9**, and **9(D)** between 600 and 1800 cm^{-1}.

fourfold and twofold deuteration in **6** and **8**, respectively. Thus, an assignment of the bands in this region for the various tetrapyrroles and their isotopomers solely based on the results of the normal-mode analysis of **5** is not possible.

On the other hand, above 1500 cm^{-1} the spectra of **5** and **7** (Figure 3) and **2** and **9** (Figure 4) are more closely related. These compounds have the same number of bands in this region, as is also clearly demonstrated by a detailed band-fitting analysis.[9, 19] The similarities also refer to the relative band intensities and, at least for some bands, to the frequencies. The modes in this region are due to stretching vibrations of the chromophore skeleton.[19] Moreover, these modes, when calculated for **5**, have a largely localized character inasmuch as their main contributions (ca. 50%) in terms of the potential energy distribution (PED) each originate from internal coordinates of an individual ring and the adjacent methine bridges. Given that the normal-mode compositions are comparable for the various tetrapyrroles, the similiarities of the vibrational band patterns can readily be understood in terms of the identical structure of the inner rings B and C.[8a, 9, 19]

Comparison of the RR spectra of the non-deuterated and the selectively deuterated tetrapyrroles now helps to identify the modes involving the corresponding C–H bending vibrations. While the in-plane coordinates are distributed among the modes in the region between 1200 and 1400 cm^{-1}, for which a detailed assignment is extremely difficult, the out-of-plane vibrations (HOOP vibrations) are expected to contribute to modes in the less crowded region between 650 and 850 cm^{-1}.[19] Hence, the spectra of the deuterated isotopomers offer the opportunity to further check previous assignments for these modes,[19] which, in addition, are of diagnostic value for the interpretation of the RR spectra of phytochrome.[8]

For **5**, the HOOP internal coordinate of the C–D methine bridge has been calculated to be localized in three modes (total: 88% PED); two of them are assigned to the RR bands at 735 and 818 cm^{-1}.[19] The spectrum of **7** also shows a band at 818 cm^{-1} of similar intensity as for **5** (Figure 5, Table 1). In both **6** and **8** this band has disappeared, which supports the assignment to a HOOP mode of the bridge C–D in **5** and **7**. The verification of the assignment of the weak 735 cm^{-1} band for **5** is less clear as it is not detected in **7**.

Table 1. Assignment of the methine bridge HOOP modes for various tetrapyrrole isotopomers [a].

Mode no.	Calcd		Measured frequencies (cm^{-1})							
	PED (%)	$\tilde{\nu}$ (cm^{-1})	**5**	**6**	**7**	**8**	**2**	**4**	**9**	**9(D)**
ν_{147}	C–D (30)	804	818	–	818	–	828	825	802	811
ν_{148}, ν_{149}	B–C (32)	803	783	785	806	802	–	–	–	–
ν_{150}	A–B (30)	781	790	–	–	–	816	–	797	797
ν_{153}	C–D (19)	735	735	–	–	–	–	–	–	–
ν_{157}	A–B (17)	703	709	–	–	–	–	–	–	–
ν_{158}	A–B (17)	696	–	–	712	–	–	–	–	–
ν_{160}	C–D (39)	686	–	–	–		–	–	–	–

[a] Mode numbering, calculated frequencies (in cm^{-1}) and PED (in %) refer to the normal-mode analysis for **5** [19].

The HOOP internal coordinates of the A–B methine bridge are also predicted to be largely localized in three modes (total: 64% PED). In **5**, one of them has been previously assigned to the weak band at ca. 699 cm^{-1}.[19] However, in view of the present data the adjacent stronger band at 709 cm^{-1}, which may correspond to the 712 cm^{-1} band for **7**, appears to be the more likely candidate, as both bands disappear in the deuterated iso-

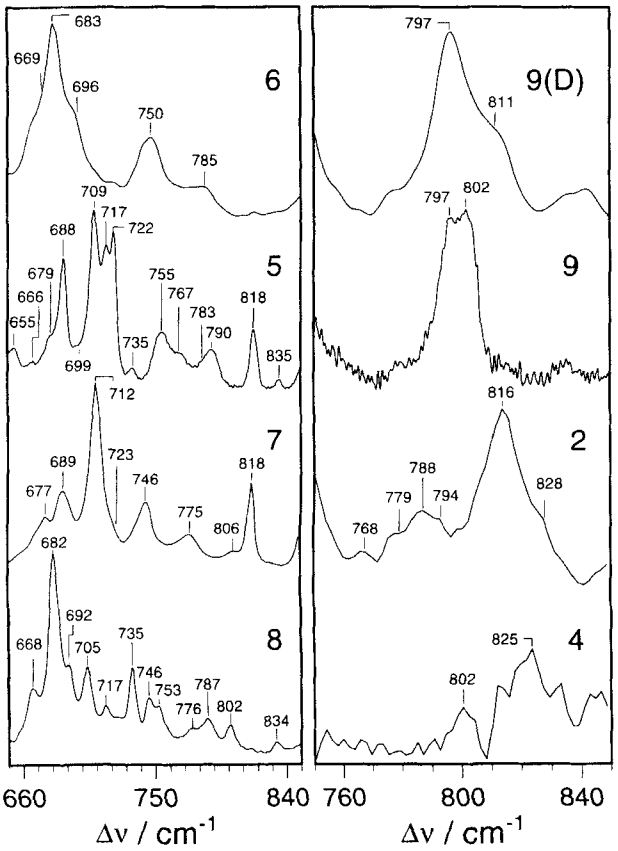

Figure 5. RR spectra in the HOOP region of **5**, **6**, **7**, and **8** (left panel) and **2**, **4**, **9**, and **9(D)** (right panel).

topomers. The second mode in **5** has been assigned to the weak band at 783 cm^{-1}, which is close to the stronger 790 cm^{-1} band attributed to the corresponding mode of the B–C methine bridge.[19] Again, these assignments should be reversed, since in **6** the latter band cannot be detected any more, whereas there is still some RR intensity at ca. 785 cm^{-1}. Consequently, the HOOP modes of the B–C bridge in **7** and **8** may be assigned to the bands at 806 and 802 cm^{-1}, respectively. The counterparts of the 783 cm^{-1} band for **5** are presumably not RR-active in **7** and **8**. The RR spectrum of **5** (but not of **7**) also displays a weak band at 835 cm^{-1}, tentatively attributed to the C_β HOOP mode of the vinyl group of ring D. The disappearance of this band in **7** also supports this assignment. The assignments of the HOOP modes were cross-checked by comparison with the spectra of the tetrapyrroles deuterated at the pyrrole nitrogens (spectra not shown). None of these bands was significantly affected by this H/D exchange, ruling out an assignment to N–H out-of-plane bending modes.

The HOOP region of the RR spectrum of **9** differs somewhat from that of **5**. Instead of a single sharp band at 818 cm^{-1} there is an asymmetric peak at a substantially lower frequency with two similarly strong and closely spaced components at 797 and 802 cm^{-1} (Figure 5). Moreover, in contrast to the 818 cm^{-1} band of **5**, the doublet at ca. 800 cm^{-1} in **9** is affected upon deuteration at the pyrrole nitrogens [**9(D)**]. Apparently, the 802 cm^{-1} band shifts to higher frequencies (811 cm^{-1}), while the low-frequency component (797 cm^{-1}) remains unchanged. The former shift indicates that, in the non-deuterated compound, the underlying mode includes HOOP vibrations not only from a methine-bridge but also, to a small extent, from an N–H group. Upon H/D exchange, the contribution of this latter vibration is removed so that the resulting mode (with a different PED) now appears at slightly higher frequencies.

In the dipotassium salt of **2** the corresponding doublet appears at 816 and ca. 828 cm^{-1}, that is, both components are shifted to higher frequencies compared to those of diester **9**. A similar frequency difference has also been observed for biliverdin (spectrum not shown) and its dimethyl ester **5**. This effect is therefore attributed to an apparently more general effect of esterification of the propionate side chains. It remains unclear whether these shifts are due to a small kinematic coupling of propionate and methine bridge HOOP vibrations, or due to subtle structural differences between the tetrapyrrole esters and salts. The latter explanation appears to be more likely, as the structure-sensitive band pattern in the region between 1520 and 1720 cm^{-1} reveals differences between **2** and **9** (Figure 4). In any case, the doublet of **2** ought to result from modes of largely HOOP character of the methine bridges. In fact, deuteration at C-5 of **2** (= formation of **4**) leads to the disappearance of the 816 cm^{-1} band. This observation supports the assignment to a HOOP mode of the A–B bridge. Although the poor signal-to-noise ratio of this RR spectrum, due to a relatively strong fluorescence background, prevents an unambiguous identification of the remaining weak RR bands, the second band at the high-frequency side appears to be largely unaffected, except for a small frequency shift down to 825 cm^{-1}. This band may therefore be due to the HOOP mode of the B–C or, more likely, of the C–D bridge, in analogy to the assignments in **5**.

Conclusions

The deuterated isotopomers prepared in this work have been shown to be particularly useful for the assignments of the methine bridge HOOP vibrations in open-chain tetrapyrroles. Moreover, the spectra of these compounds now complement the set of experimental data, to which a refinement of the calculated force fields can refer. Thus, a safer assignment of the vibrational spectra of open-chain tetrapyrroles in the entire frequency range will become possible.

Experimental Section

^1H and ^{13}C NMR spectra were obtained with a Bruker AM-400 spectrometer. Mass spectrometry was carried out using a Finnigan MAT 95 instrument. UV/Vis spectra were recorded with a Shimadzu UV-2102 PC spectrometer. IR spectra were measured with an IFS FT-IR spectrometer (Bruker). RR spectra were obtained with 1064 nm excitation using a Bio-Rad FT-Raman spectrometer equipped with a Nd-YAG laser (Spectra Physics, FC-106 V). Further details of the experimental set-ups are given elsewhere.[8a, 9, 19]

Phycocyanobilin dimethyl ester (9) was obtained by esterification of **2** following literature protocols.[2b]

5,15,18²,18²-Tetradeuteriobiliverdin IXα dimethyl ester (6): Biliverdin IXα dimethyl ester **5**[14] (31 mg, 50 μmol), dissolved in $CDCl_3$ (0.3 mL)—used as internal lock—and [D_1]trifluoroacetic acid (1 mL = 13 mmol, 99.5 atom% D), was sealed in an evacuated (3 freeze–pump–thaw cycles) NMR tube and kept at 100 °C. The reaction was followed by ^1H NMR spectroscopy (400 MHz). After 20 h, the signals at δ = 6.5–6.3, 5.1–5.0 (CH-5,15,18²) had

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mostly disappeared and heating was stopped. The solvent was evaporated, and the residue dissolved in methanol (30 mL) with 5% H_2SO_4 for esterification. The solution was stirred for 16 h in the dark, poured into ice-water (100 mL), and then extracted with CH_2Cl_2. The combined organic extracts were washed with water (4 × 100 mL) until neutral and dried over Na_2SO_4, and the solvent was removed under reduced pressure. The resulting product was chromatographed on preparative TLC (Merck silica gel, toluene–ethanol–ethyl acetate = 10:1:1).[2b] Recrystallization of the blue-green fraction ($CHCl_3$ 4% CH_3OH, hexane) afforded [D_4]diester **6** (19 mg = 61%) with a purity >98% (HPLC[21a]), m.p. 213–215 °C. ^{1}H NMR[14] (400 MHz, $CDCl_3$): δ = 8.0 (br s, 3 NH), 6.78 (s, CH-10), 6.64 (m_{ABX}, $CH_{(X)}$-3^1), 6.48 (br s, $CH_{(X)}$-18^1), 5.67 (m_{ABX}, $CH_{(B)}$-3^2), 5.63 (m_{ABX}, $CH_{(A)}$-3^2), 3.66 (s, $2OCH_3$), 2.92 (m, $2CH_2$-β), 2.57 (t, $2CH_2$-α), 2.17 (s, CH_3-13), 2.09 (s, CH_3-17), 2.07 (s, CH_3-7), 1.87 (s, CH_3-2); 85 atom% D (integrated intensities yield 89 atom% D at C-5 and C-15 and 80 atom% D at C-18^2, C-18^2); ^{13}C NMR[22] (100 MHz, $CDCl_3$): δ = 173.07 (2 ×), 171.73, 170.61, 151.92, 148.08, 142.36, 140.80, 140.36, 140.33, 139.81, 139.66, 138.37, 137.19, 129.09, 128.03, 127.29, 126.50, 125.78, 125.67, 122.58, 120.19 (br m), 114.25, 97.50 (br m, 2 ×), 51.74 (2 ×), 35.21 (2 ×), 19.87 (2 ×), 9.65, 9.51, 9.47 (2 ×); MS (70 eV): *m/z*: 614 (M^+, 100%, 56% D_4), 613 (M^+, 48%, 29% D_3); HR-MS ($C_{35}H_{34}D_4N_4O_6$): calcd. 614.3042, found 614.3038; FT-IR (CCl_4): $\tilde{\nu}$ = 2957, 2926, 2872, 1740, 1704, 1586, 1457, 1437, 1385, 1366, 1218, 1169, 1096 cm^{-1}; UV/Vis ($CHCl_3$): λ_{max} = 379 nm (ε = 51800), 660 (14300).

5,15-Dideuteriomesobiliverdin IXα dimethyl ester (8): A solution of mesobiliverdin IXα dimethyl ester **7** (15 mg, 25 μmol),[23] purified by chromatography (Merck Lobar C silica gel with toluene–ethanol–ethyl acetate = 98:1:1)[2b] in $CDCl_3$ (0.3 mL)—used as internal lock—and [D_1]trifluoroacetic acid (1 mL = 13 mmol, 99.5 atom% D), was heated in a sealed, evacuated (3 freeze–pump–thaw cycles) NMR tube. After the ^{1}H NMR signals at δ = 6.36–6.37 (CH-5,15) had mostly disappeared (100 °C, 18 h), the reaction was worked up as described for **6**. Yield 15 mg (97%), purity >98% (HPLC[21h]), m.p. 234–235 °C. ^{1}H NMR[23] (400 MHz, $CDCl_3$): δ = 8.15 (br s, 3 NH), 6.71 (s, CH-10), 3.65 (s, $2OCH_3$), 2.90 (t, J = 7.5 Hz, $2CH_2$-β), 2.53 (2 t, J = 7.5 Hz, CH_2-α), 2.49 (q, J = 7.4 Hz, CH_2-18^1), 2.26 (q, J = 7.6 Hz, CH_2-3^1), 2.08 and 2.06 (2 ×) (s, $3CH_3$-7, 13,17), 1.80 (s, CH_3-2), 1.19 (t, J = 7.6 Hz, CH_3-18^2), 1.05 (t, J = 7.6 Hz, CH_3-3^2); 97 atom% D at C-5, C-15 according to integrated intensities; ^{13}C NMR[22] (100 MHz, $CDCl_3$): δ = 173.10 (2 ×), 172.51, 172.11, 150.53, 149.49, 146.83, 141.31, 140.88, 140.67, 140.48, 139.89, 137.71, 137.41, 134.43, 128.44, 128.19, 127.83, 114.24, 95.88 (br m, 2 ×), 51.74 (2 ×), 35.24 (2 ×), 19.86 (2 ×), 17.86, 16.94, 14.44, 12.98, 9.58, 9.49 (2 ×), 8.40; MS (70 eV): *m/z* 616 (M^+, 100%, 86% D_2); HR-MS ($C_{35}H_{40}D_2N_4O_6$): calcd. 616.3230, found 616.3209; FT-IR (CCl_4): $\tilde{\nu}$ = 2967, 2934, 2870, 1743, 1729, 1700, 1677, 1609, 1584, 1437, 1240, 1195, 1145, 1091 cm^{-1}; UV/Vis ($CHCl_3$): λ_{max} = 369 nm (ε = 54600), 631 (16000).

5-Deuteriophycocyanobilin (4): Phycocyanobilin (**2**)[2] was prepared by purification on reverse-phase chromatographic column (Merck Lobar A[9b] Lichropräp. C_{18} 40–20 μm with CH_3CN:potassium phosphate buffer (7.2 mM, pH 7.8) = 28:72) followed by acidification with KOAc buffer (4 M, pH 4.8), extraction into $CHCl_3$/1% CH_3OH and precipitation with hexane.[12] Pure **2** (15 mg, 26 μmol) was dissolved under Ar bubbling in $CDCl_3$ (0.3 mL)—used as internal lock—and [D_1]trifluoroacetic acid (1 mL = 13 mmol, 99.5 atom% D, Ar saturated) in an NMR tube. After the ^{1}H NMR signal at δ = 6.0 (CH-5) had mostly disappeared (25 °C, 2.5 h), the solvent was removed at 0 °C with a vacuum pump. The residue was taken up in D_2O (2 mL) and [D_5]pyridine (0.5 mL) and extracted with $CHCl_3$/2% CH_3OD (5 × 10 mL). The combined organic layers were washed with D_2O (2 × 2 mL) until the aqueous wash was neutral. The organic layer was dried over Na_2SO_4, and the organic solvents were removed on a rotary evaporator. The potassium salts of **2** and **4** were obtained after dissolving the compounds in CH_3CN/potassium phosphate buffer[21c] and evaporation of the solvent. After recrystallization from $CHCl_3$/1% CH_3OH/hexane, **4** was collected.[12] Yield: 14 mg (93%), purity >98% (HPLC[21c]), m.p. >300 °C. ^{1}H NMR[16] (400 MHz, [D_5]pyridine): δ = 8.20 (br s, 3 NH), 7.28 (s, CH-10), 6.31 (q, J = 6.5 Hz, CH-3^1), 6.07 (s, CH-15), 3.35 (q, J = 7.7 Hz, CH-2), 3.19 (t, J = 6.9 Hz, CH_2-12^1), 3.11 (t, J = 7.2 Hz, CH_2-8^1), 2.86 (q, J = 7.2 Hz, $2CH_2$-8^2,12^2), 2.49 (m, CH_2-18^1), 2.14 (s, CH_3-13), 2.09 (s, CH_3-17), 2.02 (s, CH_3-7), 1.70 (d, J = 7.3 Hz, CH_3-3^2), 1.49 (d, J = 7.0 Hz, CH_3-2), 1.25 (t, J = 7.5 Hz, CH_3-18^2); 92 atom% D at C-5 according to integrated intensities; ^{13}C NMR (100 MHz, [D_5]pyridine): δ = 177.94, 175.29 (2 ×), 174.89, 165.00, 148.37, 145.91, 142.56, 141.46, 139.14, 136.79, 134.98, 133.95, 132.87, 132.36, 131.70, 122.63, 112.56, 96.05, 87.62 (br m), 38.28, 36.46, 36.30, 20.61, 20.44, 17.54, 15.91, 14.75, 13.35, 9.67, 9.60, 9.26; MS (ESIp): *m/z*: 588 (M+H$^+$, 100%, 74% D_1), 611 (M+Na$^+$, 75%), 1176 (2M+2H)$^+$, 30%), 1198 (2M+H+Na)$^+$, 35%; **2** (reference): *m/z*: 587[18,24] (M+H$^+$, 100%), 610 (M+Na$^+$, 75%), 1174 (2M+2H)$^+$, 40%), 1196 (2M+H+Na)$^+$, 35%); FT-IR (CCl_4) FT-IR (KBr): $\tilde{\nu}$ = 2962, 2924, 2855, 1701, 1597, 1539, 1455, 1388, 1312, 1247, 1233, 1220, 1161, 1108, 1065, 964 cm^{-1}; UV/Vis (CH_3OH): λ_{max} = 364 nm (ε = 51500), 604 (17100).

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