Monatshefte für Chemie 131, 879–888 (2000)

Monatshefte für Chemie
Chemical Monthly

Synthesis of a Model for Phycocyanin with Respect to the Acid-Base Chemistry between Protein and Chromophore

Michael Stanek[1,2,*] and **Karl Grubmayr**[1]

[1] Institut für Chemie, Johannes-Kepler-Universität, A-4040 Linz, Österreich
[2] DSM Fine Chemicals Austria GmbH, St.-Peter-Strasse 25, A-4021 Linz, Österreich

Summary. Earlier investigations have shown that the cysteine adduct of phycocyanobilin dimethylester qualifies for intramolecular protonation [1]. In this context, the -Cys-Ala-Phe-Asp-tetrapeptide adduct of phycocyanobilin dimethylester was synthesized and examined for its protonation properties with respect to the zwitterionic interaction between chromophore and protein.

Keywords. Phycocyanobilin; Tetrapeptide; Protonation; Zwitterions; Chromophores.

Introduction

Open chain 2,3-dihydrobilindiones such as phycocyanobilin [2, 3] or phytochromobilin [4, 5] serve as prosthetic groups in antenna pigments of cyanobacteria or as photoreceptors in the photomorphogenesis in higher plants. From crystal structures of phycocyanin [6] as well as of other phycobiliproteins [7] such as allophycocyanin [8], phycoerythrocyanin [9], and phycoerythrin [10, 11], a common principle becomes evident: all chromophores are covalently linked to the apoprotein by thioethers and adopt a stretched (all*Z*, 5*anti*, 10*syn*, 14*anti*)-geometry adjacent to their α-helical protein segment due to the formation of various salt bridges (Fig. 1). Generally, these stretched conformations are responsible for their distinct biologically important photophysical and photochemical properties [12–18]. However, 2,3-dihydrobilindione chromophores prefer a helical (all*Z*, 5*anti*, 10*syn*, 14*anti*)-geometry in solution except when hexamethylphosphoric triamide (*HMPT)* is used as solvent [19].

The protonation of 2,3-dihydrobilindion chromophores, intermolecular as well as intramolecular, always takes place at the nitrogen atom of the azafulvenic ring B moiety [1]. Considering phycocyanin, it is exclusively caused by the highly conserved aspartic acid residue. Protonation of bilindiones is indicated best by significant hyperchromic and bathochromic shifts of the long-wavelength absorption maxima and can easily be detected by measuring their UV/Vis-spectra [20–22].

As shown recently by the synthesis of two diastereomers of a cysteine adduct of phycocyanobilin dimethylester [1, 23], intramolecular protonation is entropically

* Corresponding author

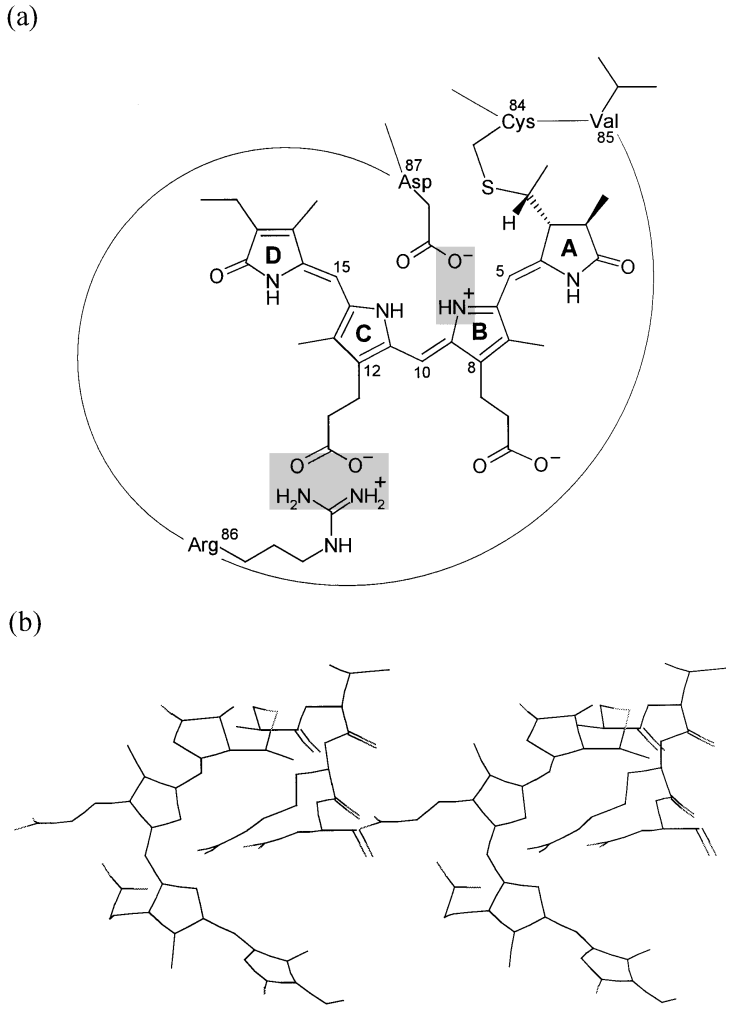

Fig. 1. (a) Extended (5*anti*, 10*syn*, 14*anti*) conformation of the protein bound phycocyanobilin chromophore; (b) surroundings of the α-helical peptide segment -Cys84-Val-85-Arg86-Asp87- of phycocyanin from *Fremyella Diplosiphon* [6] (stereo view of the wire frame model)

controlled and proceeds quantitatively at low temperatures, even if the protonated chromophore is slightly more acidic ($pK_a = 4.6$) [20, 21] than the carboxylic acid of cysteine which can be compared to acetic acid ($pK_a = 4.76$). Here we report on the synthesis, the structure, and the spectroscopic properties of the model compound **8**, which is in equilibrium with its zwitterionic species $\mathbf{8}^{\pm}$, representing the adduct of the tetrapeptide of N-benzyloxycarbonyl-cysteinyl-alanyl-phenylalanyl-(α-methyl)-aspartate (*Z*-Cys-Ala-Phe-Asp-OMe) and phycocyanoblin dimethylester (*PCBDME*) in its (2*R*,3*R*,3′*R*,Cys*R*,Ala*S*,Asp*S*)-configuration. The tetrapeptide corresponds to the consensus sequence of phycocyanines (-Cys-*Xxx*-Arg-Asp-) [24] with the exception of phenylalanine and alanine, whereas alanine has been found in many phycobiliprotein subunits at this position as well. Arginine was replaced by phenylalanine due to the difficulty of handling the propionic acid residues of phycocyanobilin separately, one of which would probably support the formation of a

short α-helix by the arrangement of a salt bridge between arginine and the propionic acid in position 12 of phycocyanobilin (Fig. 1). Concerning the α-helix propensity of amino acids, phenylalanine might show a tendency towards the formation of α-helices, as was deduced from statistical analysis of protein X-ray structures [25]. On the other hand, refined statistical analysis on position-specific preferences clearly indicated that the conformational propensity of amino acids are strongly position-dependent [26]. Nevertheless, **8** is able to mimic the acid-base chemistry between protein and chromophore under certain conditions due to the possibility of an entropically controlled intramolecular protonation. However, a change of the helical conformation of the chromophore to the stretched (5*anti*, 10*syn*, 14*anti*)-arrangement was not observed. The formation of a salt bridge between the nitrogen atom of the azafulvenic ring B moiety and the aspartic acid residue of the tetrapeptide seems to provide insufficient driving force for changing the overall geometry.

Results and Discussion

Synthesis

The synthesis of the cysteine adduct of phycocyanobilin dimethylester with (2*R*,3*R*,3′*R*,Cys*R*)-configuration was performed according to Ref. [1]. Since *Carpino*'s report on peptide coupling with 9-fluorenylmethyloxycarbonyl (F*moc*) amino acid halogenides occuring without racemization, a convenient method of peptide synthesis in a two-layer system is at hand [27, 28]. Compound **6** was synthesized following this approach (Fig. 2).

The final coupling step towards the tetrapeptide **7** was accomplished using the uronium salt O-(7-azabenzotriazole-1-yl)-N,N,N′,N′,-tetramethyluronium-hexafluoro-

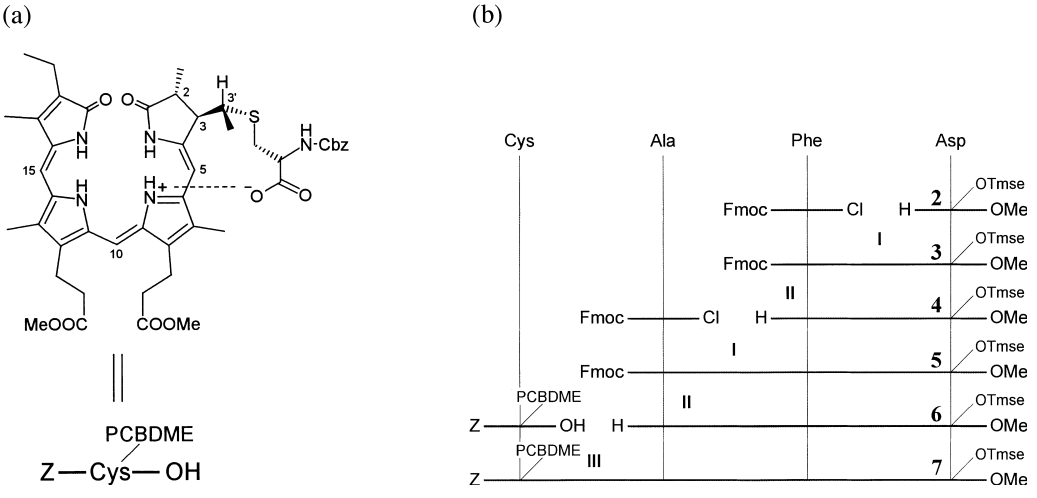

Fig. 2. (a) The phycocyanobilin dimethyl ester chromophore in its helical (5*syn*, 10*syn*, 14*syn*)-conformation can be interpreted as a protecting group for cysteine; the compound is drawn in its zwitterionic form and exists at room temperature in equilibrium with its neutral form (Ref. [1]); (b) reaction scheme for the synthesis of model compound **7**: I) $CHCl_3$, 40 min, II), $CHCl_3$, 4-(aminomethyl)-piperidine, 90 min, H*ATU*, HOAt, *DIPEA*, *DMF*

phosphate (H*ATU*), the peptide coupling additive 1-hydroxy-7-azabenzotriazole (HO*At*) and diisopropylethylamine (*DIPEA*) in *DMF* [29, 30]. After deprotection of the trimethylsilylethyl (*Tmse*) protecting group with tetra-*n*-butylammonium fluoride (*TBAF*) in *THF* and dilution with CH_2Cl_2 followed by treatment with aqueous $NaHCO_3$, aqueous HCl, H_2O (3×), and column chromatography on silica gel, compound **8** with (4*Z*,9*Z*,15*Z*,2*R*,3*R*,3′*R*,Cys*R*,Ala*S*,Phe*S*,Asp*S*)-configuration was obtained. As a mater of fact, intermolecular protonated 2,3-dihydrobilindione chromophores dissolved in CH_2Cl_2 or $CHCl_3$ are changed to their neutral form by vigorous shaking of the corresponding solution with H_2O; it can therefore be deduced with certainty that the aspartic acid residue in **8** prevails as carboxylic acid and not as carboxylate.

Intramolecular protonation

By addition of the stoichiometric amount of acetic acid to the *Tmse* protected compound **7** dissolved in chloroform, intermolecular protonation was not observed due to the 2,3-dihydroblindione chromophore which is slightly more acidic than acetic acid (see above). However, addition of a 4900-fold molar excess of acetic acid and cooling to −54°C led to a minor change of the long-wavelength absorption band (Fig. 3a).

The model compound **8** dissolved in chloroform only shows a small shoulder around 670 nm in the UV/Vis spectra which might result from the intramolecular protonation between the aspartic acid residue and the nitrogen atom of the azafulvenic ring B moiety. This may be inferred from the disappearance of the shoulder upon addition of *DIPEA* and, therefore, the formation of the diisopropyl ethylammonium carboxylate of **8**. In order to intesify and to prove this characteristic – especially since no impressive change of the long-wavelength absorption band could be detected by cooling or heating –, a 2800-fold molar excess of acetic acid was added. The use of acetic acid should not be interpreted as an initiation of intermolecular protonation of the chromophore in this case, but is meant to change the solvent polarity, inducing a slight change of the arbitrary peptide conformation and effecting a closer distance beetween the aspartic acid residue and the nitrogen atom of the azafulvenic ring B moiety. Actually, a batho- and hyperchromic shift of the long-wavelength absorption band is observed at room temperature, which can reversibly be extended to a ratio of approximately 1:1 between the long and short-wavelength absorption bands by cooling to −54°C (Fig. 3b). Assuming that the pK_a values of acetic acid and the carboxyl group of aspartic acid are more or less equal, and knowing that the protection of a carboxyl group, like that of **7**, leads to a neglectable hyperchromicity of the long-wavelength absorption band upon addition of acetic acid, the proton transfer in **8** must proceed intramolecularly, resulting in the formation of the zwitterion $\mathbf{8}^{\pm}$. Unfortunately, the equilibrium is not quantitatively shifted towards $\mathbf{8}^{\pm}$ at −54°C in chloroform as deduced from a slight shift of the absorption maximum upon further cooling.

The desired change of the helical conformation of the chromophore to the stretched (5*anti*, 10*syn*, 14*anti*)-arrangement which would also presuppose a short α-helix formation of the peptide, was not observed. This change would easily have been detected by a multiple enhancement of the long-wavelength absorption band compared to its short-wavelength counterpart.

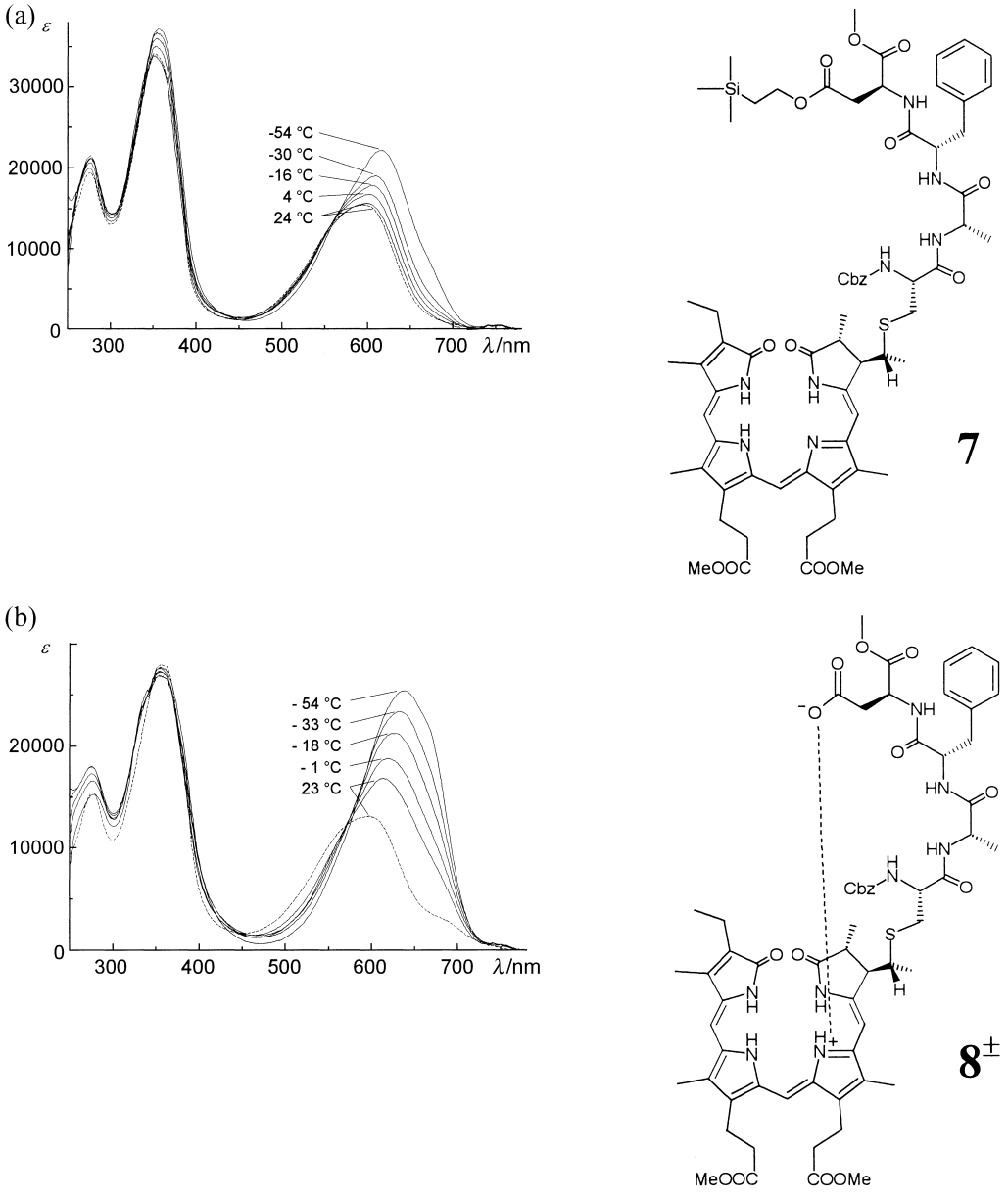

Fig. 3. (a) UV/Vis spectrum of **7** (dashed line) in $CHCl_3$ ($c = 2.4 \cdot 10^{-5} M$); spectra with solid lines recorded at different temperatures after the addition of a 4900-fold molar excess of acetic acid; (b) UV/Vis specta showing the temperature dependence of the proton transfer equilibrium of **8** and $\mathbf{8}^{\pm}$ in $CHCl_3$ ($c = 2.7 \cdot 10^{-5} M$): spectra with solid lines recorded at different temperatures after the addition of a 2800-fold molar excess of acetic acid, spectrum with dashed line: measured without acetic acid; all values of ε are corrected to room temperature

Although the overall change of the geometry does not happen, a conclusion may be drawn for phycobiliproteins: intramolecular protonation by aspartic acid residues is favoured by the highly ordered arrangement of consensus sequences enabling the correct geometry necessary for effective ion-pair formation.

Experimental

All chemicals were of reagent grade. Solvents were generally distilled prior to use, *THF* was distilled from sodium benzophenone ketyl. Column chromatography was performed on silica gel (E. Merck, silica gel 60, 0.63–0.200 mm). NMR spectra were recorded either on a Bruker Avance DRX-500 or on a Bruker WM-360 instrument. The assignment of ^{1}H and ^{13}C signals is based on gradient-enhanced HMQC, gradient-enhanced phase sensitive DQF-COSY, and ROESY experiments. UV/Vis and CD spectra were recorded on a Hitachi U-3210 spectrometer and a Jobin-Yvon Mark V circular dichograph. The electrospray mass spectrum of **8** was measured on a Hewlett Packard MS-Engine 5989 API.

(S)-N-Benzyloxycarbonyl-β-(2-trimethylsilylethyl)-α-methyl-aspartate (**1**; $C_{18}H_{27}NO_6Si$)

According to Ref. [31], 1.4 cm^3 of pyridine and 1.45 cm^3 of 2-trimethylsilyl-ethanol were added to a solution of (*S*)-N-benzyloxycarbonyl-α-methyl-aspartate (2.43 g, 8.6 mmol) in 8.6 cm^3 acetonitrile. After cooling in an ice bath, 0.3 g 1-hydroxybenzotriazole (HO*Bt*) and 1.9 g dicyclohexyl-carbodiimide (*DCC*) were added. This solution was stirred at 0°C for 2 h and kept in the refrigerator over night.

Oxalic acid (0.5 cm^3 5 *M* in *DMF*) was added to the reaction mixture which was stirred for another 40 min and then filtered by suction. The filtrate was diluted with 50 cm^3 of CH_2Cl_2, washed with 100 cm^3 of 0.1 N aqueous HCl, 100 cm^3 of 0.2 M aqueous $NaHCO_3$, and dried over Na_2SO_4. The organic phase was evaporated to give 72% of **1** as a clear oil.

^{1}H NMR (360 MHz, $CDCl_3$): $\delta = 7.35$ (m, 5H, phenyl-H), 5.78 (broad d, 1H, J(NH, $^{\alpha}$CH) = 8.3 Hz, Asp-NH), 5.12 (s, 2H, benzyl-CH_2), 4.62 (ddd, 1H, Asp-$^{\alpha}$CH), 4.17 (t, 2H, -O-CH_2-), 3.75 (s, 3H, -O-CH_3), 3.01 (dd, 1H, J($^{\beta}$CH(1),$^{\alpha}$CH) = 4.4 Hz, J($^{\beta}$CH(1),$^{\beta}$CH(2)) = 17.1 Hz, Asp-$^{\beta}$CH(1)), 2.82 (dd, 1H, J($^{\beta}$CH(2),$^{\alpha}$CH) = 4.5 Hz, J($^{\beta}$CH(2),$^{\beta}$CH(1)) = 17.1 Hz, Asp-$^{\beta}$CH(2)), 0.97 (t, 2H, -CH_2-Si-), 0.03 (s, 9H, -Si(CH_3)$_3$) ppm.

(S)-β-(2-Trimethylsilylethyl)-α-methyl-aspartate (**2**; $C_{10}H_{21}NO_4Si$)

According to Ref. [32], **1** (1.1 g, 2.8 mmol) was dissolved in 5 cm^3 of isopropanol. Palladium on activated carbon (0.1 g) was added to the stirred solution, and the amino acid was decarboxylated by hydrogenation in a flask with a hydrogen-filled balloon. The catalyst was filtered off, and the alcohol was evaporated in vacuum to yield **2** quantitatively as a clear oil.

^{1}H NMR (360 MHz, $CDCl_3$): $\delta = 4.20$ (t, 2H, -O-CH_2-), 4.62 (m, 1H, Asp-$^{\alpha}$CH), 3.79 (S, 3H, -O-CH_3) 3.01 (dd, 1H, J($^{\beta}$CH(1), $^{\alpha}$CH) = 4 Hz, J($^{\beta}$CH(1),$^{\beta}$CH(2)) = 17.1 Hz, Asp-$^{\beta}$CH(1)), 2.89 (dd, 1H, J($^{\beta}$CH(2),$^{\alpha}$CH) = 6.3 Hz, J($^{\beta}$CH(2),$^{\beta}$CH(1)) = 17.1 Hz, Asp-$^{\beta}$CH(2)), 0.99 (t, 2H, -CH_2-Si-), 0.04 (s, 9H, -Si(CH_3)$_3$) ppm.

(PheS, AspS)-N-9-Fluorenylmethyloxycarbonyl-phenylalanyl-β-(2-trimethylsilylethyl)-α-methyl-aspartate (**3**; $C_{34}H_{40}N_2O_7Si$)

According to Refs [27, 28], to 0.68 g (2.75 mmol) of **2** in 27 cm^3 $CHCl_3$ a solution of N-9-fluorenyl-methyloxycarbonyl-phenylalanine acid chloride [27] (F*moc*-Phe-Cl) (1.22 g, 3.03 mmol) in 25 cm^3 of $CHCl_3$ and 27 cm^3 of aqueous Na_2CO_3 (5%) were added. The two-phase mixture was stirred vigorously for 40 min. The reaction mixture was diluted with 100 cm^3 of CH_2Cl_2 and 60 cm^3 of H_2O, adjusted to *pH* 2–3 by addition of aqueous HCl (0.1 *N*), washed with 100 cm^3 of H_2O, and dried over Na_2SO_4. After evaporation of the solvent the residue was purified by column chromatography (silica gel, CH_2Cl_2/EtOAc = 18/1) yielding **3** (1 g, 60%) as a slightly yellowish oil.

$R_f = 0.46$ (silica gel, CH_2Cl_2/EtOAc = 12/1); ^{1}H NMR (360 MHz, $CDCl_3$): $\delta = 7.36$ (m, 2H, 4,5-fluorenyl-H), 7.54 (m, 2H, 1,8-fluorenyl-H), 7.40 (m, 2H, 3,6-fluorenyl-H), 7.30 (m, 2H, 2,7-fluorenyl-H) 7.29−7.15 (m, 5H, phenyl-H), 6.92 (broad d, 1H, J(NH,$^{\alpha}$CH) = 7.1 Hz,Asp-NH), 5.46 (broad d, 1H, J(NH, $^{\alpha}$CH = 6.7 Hz, Phe-NH), 4.80 (ddd, 1H, Asp-$^{\alpha}$CH), 4.41 (dd, 1H, fluorenyl-CHH(1)), 4.32 (m, 1H, 9-fluorenyl-H), 4.19 (dd, 1H, fluorenyl-CH(2)H), 4.12 (t, 2H, -O-CH_2-), 3.71 (s, 3H, -O-CH_3), 3.10 (m, 2H, Phe-$^{\beta}CH_2$), 2.98 (dd, 1H, J($^{\beta}$CH(1),$^{\alpha}$CH) = 4.2 Hz, J($^{\beta}$CH(1), $^{\beta}$CH(2)) = 17.3 Hz, Asp-$^{\beta}$CH(1)), 2.77 (dd, 1H, J($^{\beta}$CH(2),$^{\alpha}$(CH) = 4.5 Hz, J($^{\beta}$CH(2), $^{\beta}$(CH)(1)) = 17.3 Asp-$^{\beta}$CH(2)), 0.94 (t, 2H, -CH_2-Si-), 0.02 (s, 9H, -Si$(CH_3)_3$) ppm. ^{13}C NMR (90 MHz, $CDCl_3$): δ = 170.87, 170.63, 170.57 (C=O), 155.70 (C=O), 143.75 143.67, 141.22, 136.03 (quaternary carbons), 129.23 (3-, 5-phenyl-CH), 128.90 (2-, 6-phenyl-CH), 127.65 (3-, 6-fluorenyl-CH), 127.00 (2-, 7-fluorenyl-CH and 4-phenyl-CH), 125.00 (1-, 8-fluorenyl-CH), 119.91 (4-, 5-fluorenyl-CH), 67.08 (fluorenyl-CH_2), 63.38 (-O-CH_2-), 55.79 (Phe-$^{\alpha}$CH), 52.67 (-O-CH_3), 48.55 (Asp-$^{\alpha}$CH), 47.04 (9-fluorenyl-CH), 38.49 (Phe-$^{\beta}CH_2$), 36.20 (Asp-$^{\beta}CH_2$), 17.19 (-CH_2-Si-), -1.62 (-Si$(CH_3)_3$) ppm.

(PheS,AspS)-Phenylalanyl-β-(2-trimethylsilylethyl)-α-methyl-aspartate (**4**; $C_{19}H_{30}N_2O_5Si$)

According to Refs. [27, 28], **3** (0.89 g, 1.4 mmol) was dissolved in 25 cm^3 of $CHCl_3$ and treated with 5 cm^3 of 4-(aminomethyl)-piperidine. After 90 min, 70 cm^3 of $CHCl_3$ were added, and the organic phase was washed with two 100 cm^3 portions of saturated NaCl solution and with three 100 cm^3 portions of phosphate buffer of $pH = 5.5$ (prepared from 90 g of $KH_2PO_4 \cdot H_2O$ and 32.7 g of Na_2HPO_4 in 500 ml of deionized water). The organic phase was dried over Na_2SO_4 and evaporated to give 0.57 g of a mixture of **4** and the corresponding diketo-piperazine by-product ((3*S*,6*S*)-trimethylsilylethyl-(3-(2,5-dioxo-6-benzyl)-piperazinly)-ethanoat; the by-product was isolated by column chromatography after preparation of **5**).

^{1}H NMR (360 MHz, $CDCl_3$): δ = 8.15 (broad d, 1H, J(NH, $^{\alpha}$CH) = 8.5 Hz, Asp-NH), 7.35-7.15 (m, 5H, Phenyl-H), 4.86 (ddd, 1H, Asp-$^{\alpha}$CH), 4.16 (m, 2H, -O-CH_2-), 3.74 (s, 3H, -O-CH_3), 3.66 (dd, 1H, J($^{\alpha}$CH,$^{\beta}$CH(1)) = 3.8 Hz, J($^{\alpha}$CH,$^{\beta}$CH(2)) = 4.21 Hz, Phe-$^{\alpha}$CH), 3.22 (dd, 1H, J($^{\beta}$CH(1), $^{\alpha}$CH) = 3.8 Hz, J($^{\beta}$CH(1),$^{\beta}$CH(2)) = 13.7 Hz, Phe-$^{\beta}$CH(1)), 2.99 (dd, 1H, J($^{\beta}$CH(1),$^{\alpha}$CH) = 4.7 Hz, J($^{\beta}$CH(1),$^{\beta}$CH(2)) = 17.1 Hz, Asp-$^{\beta}$CH(1)), 2.76 (dd, 1H, J($^{\beta}$CH(2),$^{\alpha}$CH) = 4.6 Hz, J($^{\beta}$CH(2),$^{\beta}$CH(1) = 17.1 Asp-$^{\beta}$CH(2)), 2.72 (dd, 1H, J($^{\beta}$CH(2)) = 4.2 Hz, J($^{\beta}$CH(2),$^{\beta}$CH(1)) = 13.7 Hz, Phe-$^{\beta}$CH(2)), 0.97 (t, 2H, -CH_2-Si-), 0.04 (s, 9H, -Si$(CH_3)_3$) ppm.

(AlaS,PheS,AspS)-N-9-Fluorenylmethyloxycarbonyl-alanyl-phenylalanyl-β-(2-trimethylsilylethyl)-α-methyl-aspartate (**5**; $C_{37}H_{45}N_3O_8Si$)

5 was synthesized according to the protocol described for the synthesis of **3** employing N–9-fluorenylmethyloxycarbonyl-alanine acid chloride [27] (F*moc*-Ala-Cl). The residue was purified by column chromatography (silica gel, CH_2Cl_2/MeOH = 20/1) yielding **5** (0.44 g, 44%) as a yellowish oil.

$R_f = 0.75$ (silica gel, CH_2Cl_2/MeOH = 15/1); ^{1}H NMR (360 MHz, $CDCl_3$): $\delta = 7.76$ (m, 2H, 4,5-fluorenyl-H), 7.58 (m, 2H, 1,8-fluorenyl-H), 7.40 (m, 2H, 3,6-fluorenyl-H), 7.31 (m, 2H, 2,7-fluorenyl-H) 7.28-7.16 (m, 5H, phenyl-H), 6.95 (broad) d, 1H, J(NH,$^{\alpha}$CH, = 7.6 Hz,Asp-NH), 6.72 (broad d, 1H, J(NH,$^{\alpha}$CH = 6.8 Hz, Phe-NH), 5.41 (broad d, 1H, J(NH,$^{\alpha}$CH) = 6.7 Hz, Ala-NH), 4.79 (ddd, 1H, Asp-$^{\alpha}$CH), 4.73 (ddd, 1H, Phe-$^{\alpha}$CH), 4.37 (m, 2H, fluorenyl-CHH(1) and Ala-$^{\alpha}$(CH), 4.24 (m, 1H, 9-fluorenyl-H), 4.19 (dd, 1H, fluorenyl-CH(2)H), 4.13 (t, 2H, -O-CH_2-), 3.71 (s, 3H, -O-CH_3), 3.14 (dd, 1H, J($^{\beta}$CH(1),$^{\alpha}$CH) = 6.5 Hz, J($^{\beta}$CH(1)$^{\beta}$CH(2)) = 14 Hz, Phe-$^{\beta}$CH(1)), 3.06 (dd, 1H, J($^{\beta}$CH(2),$^{\alpha}$CH) = 6.6 Hz, J($^{\beta}$CH(2),$^{\beta}$CH(1)) = 14 Hz, Phe-$^{\beta}$CH(2)), 2.93 (dd, 1H, J($^{\beta}$CH(1), $^{\alpha}$(CH) = 4.4 Hz, J($^{\beta}$CH(1),$^{\beta}$CH(2)) = 17.2 Hz,Asp-$^{\beta}$CH(1)), 2.74 (dd, 2H, J($^{\beta}$(CH(2),$^{\beta}$CH(1)) = 17.2 Hz, Asp-$^{\beta}$CH(2)), 1.33 (d, 3H, J($^{\beta}CH_3$,$^{\alpha}$CH) = 6.5 Hz Ala-$^{\beta}CH_3$)), 0.94 (t, 2H, -CH_2-Si-), 0.02 (s, 9H, -Si$(CH_3)_3$) ppm; ^{13}C NMR (90 MHz, $CDCl_3$): δ = 172.11, 170.81, 170.67, 170.31 (C=O),

155.89 (C=O), 143.79, 141.25, 136.03, (quaternary carbons atoms), 129.30 (3-, 5-phenyl-CH), 128.48 (2-,6-phenyl-CH), 127.68 (3-, 6-fluorenyl-CH), 127.03 (2-, 7-fluorenyl-CH) 126.92 (4-phenyl-CH), 125.06 (1-, 8-fluorenyl-CH), 119.93 (4-, 5-fluorenyl-CH), 67.10 (fluorenyl-CH_2), 63.37 (-O-CH_2-), 54.08 (Phe-$^{\alpha}$CH), 52.66 (-O-CH_3), 50.47 (9-fluorenyl-CH), 48.49 (Asp-$^{\alpha}$CH), 47.08 (Ala-$^{\alpha}$CH), 38.19 (Phe-$^{\beta}CH_2$), 36.22 (Asp-$^{\beta}CH_2$), 18.68 (Ala-$^{\beta}CH_3$), 17.22 (-CH_2-Si-), −1.59 (-Si(CH_3)$_3$) ppm.

(AlaS,PheS,AspS)-Alanyl-phenylalanyl-β-(2-trimethylsilylethyl)-α-methyl-aspartate
(**6**; $C_{22}H_{35}N_3O_6Si$)

6 was synthesised according to the protocol described for the synthesis of **4** in a yield of 98%.

^{1}H NMR (360 MHz, $CDCl_3$): δ = 7.70 broad d, 1H, J(NH,$^{\alpha}$CH, = 8.3 Hz, Phe-NH), 7.36-7.19 (m, 5H, phenyl-H) 6.99 broad d, 1H, J(NH,$^{\alpha}$CH = 8.0 Hz, Asp-NH), 4.78 (ddd, 1H, Asp-$^{\alpha}$CH), 4.67 (ddd, 1H, Phe-$^{\alpha}$CH), 4.13 (m, 2H, -O-CH_2-), 3.71 (s, 3H, -O-CH_3) 3.44 (dd. 1H, J($^{\alpha}$CH,$^{\beta}CH_3$) = 7 Hz, Ala$^{\alpha}$CH),3.18 (dd, 1H, J($^{\beta}$CH(1),$^{\alpha}$CH) = 6.2 Hz, J($^{\beta}$CH(1)$^{\beta}$CH(2)) = 14 Hz, Phe-$^{\beta}$CH(1)), 3.04 (dd, 1H, J($^{\beta}$CH(2),$^{\alpha}$CH) = 7.6 Hz, J($^{\beta}$CH(2),$^{\beta}$CH(1)) = 14 Hz, Phe-$^{\beta}$CH(2)), 2.94 (dd, 1H, J($^{\beta}$CH(1), $^{\alpha}$(CH) = 4.5 Hz, J($^{\beta}$CH(1),$^{\beta}$(CH)(2) = 17.1 Hz, Asp-$^{\beta}$CH(1)), 2.76 (dd, 1H, J($^{\beta}$CH(2),$^{\alpha}$CH(2) = 4.8 Hz, J($^{\beta}$(CH(2),$^{\beta}$CH(1)) = 17.1 Hz, Asp-$^{\beta}$CH(2)), 0.95 (t, 2H -CH_2-Si-), 0.02 (s, 9H, -Si(CH_3)$_3$) ppm; ^{13}C NMR (90 MHz, $CDCl_3$): δ = 175.73, 170.78, 170.73 (C=O), 136.43 (1-phenyl-C) 129.27 (3-, 5-phenyl-CH), 128.38 (2-, 6-phenyl-CH), 126.76 (4-phenyl-CH), 63.32 (-O-CH_2-), 53.57 (Phe-$^{\alpha}$CH), 52.63 (-O-CH_3), 50.58 (Ala-$^{\alpha}$CH), 48.49 (Asp-$^{\alpha}$CH), 37.81 (Phe-$^{\beta}CH_2$), 36.22 (Asp-$^{\beta}CH_2$), 21.31 (Ala-$^{\beta}CH_3$) 17.17 (-CH_2-Si-), −1.63 (-Si(CH_3)$_3$) ppm.

(4Z,9Z,15Z,2R,3R,3′R,CysR,AlaS,PheS,AspS)-3-(1-(N-Benzyloxycarbonyl-cysteinyl-alanyl-phenylalanyl-β-(2-trimethylsilylethyl)-α-methyl-aspartyl-S-yl)-ethyl)-18-ethyl-2,3-dihydro-8,12-bis-(2-methoxycarbonylethyl)-2,7,13,17-tetramethyl-23H-bilin-1,19-(21H,24H)-dione
(**7**; $C_{68}H_{88}N_8O_{15}SSi$)

A solution of **6** (16 mg, 33 μmol) in 0.5 cm^3 of *DMF* (0.5 ml) and a solution of *HATU* (9 mg, 22 μmol), HO*At* (3 mg, 22 μmol), and *DIPEA* (4 μl 3 mg) in 0.5 cm^3 of *DMF* were added to a stirred solution of (4*Z*,9*Z*,15*Z*,2*R*,3*R*,3′*R*,Cys*R*)-3-(1-(N-benzyloxycarbonyl-cystein-S-yl)-ethyl)-18-ethyl-2,3-dihydro-8,12-*bis*-(2-ethoxycarbonylethyl)-2,7,13,17-tetramethyl-23*H*-bilin-1,19-(21*H*,24*H*)-dione (**1**$^{\pm}$ in Ref. [1]; 10 mg, 11.8 μmol) in 4 cm^3 of *DMF*. The reaction mixture was stirred under Ar at 25°C for 2 h, diluted with 80 cm^3 of CH_2Cl_2, washed with 0.5 *N* aqueous HCl and 2% aqueous $NaHCO_3$, and dried over Na_2SO_4. After evaporation of the solvent the residue was purified by column chromatography (silica gel, CH_2Cl_2/MeOH = 30/1) to afford 14.6 mg (96%) of **7** as a blue solid.

M.p.: 81°C; R_f = 0.36 (silica gel, CH_2Cl_2/MeOH = 30/1); ^{1}H NMR (500 MHz, $CDCl_3$; two different conformations of **7** in a ratio of 68.32 were observed at 298 K; *Bzl* = benzyl): δ = 10.41 broad s, 1H, 32% chromophore-NH), 8.74 (d, 1H, J(NH,$^{\alpha}$CH, = 4.5 Hz, 1st Cys-NH), 8.10 (d, 1H, J (NH,$^{\alpha}$CH) = 3.5 Hz, 2nd Cys-NH), 6.88 (broad s, 1H, 32% chromophore-NH), 7.39-6.97 (m, 19H, 1st Ala-NH, 1st and 2nd Phe-phenyl-H, 1st Asp-NH, 1st and 2nd 3,4,5-*Bzl*-H, 1st Phe-NH), 6.96-6.80 (m, 5H, 1st and 2nd/1,6-*Bzl*-H, 2nd Ala-NH), 6.72 (s, 1H, 1st 10-H), 6.69 (d, 1H, 2nd Asp-NH), 6.70 (s, 1H, 2 10-H), 6.56 (d, 1H, J(NH,$^{\alpha}$CH) = 7.5 Hz, 2nd/Phe-NH), 5.99 (s, 1H, 1st 15-H), 5.98 (s, 1H, 2nd 15-H), 5.59 (s, 1H, 1st 5-H), 5.42 (s, 1H, 2nd 5-H), 4.98 (d, 1st part of the 1st AB-system, 1H, J(CHH,CHH) = 12.5 Hz, *Bzl*-CHH-), 4.81 (ddd, 1H, 1st Asp-$^{\alpha}$CH), 4.75 (d, 1st part of the 2nd AB-system, 1H, *Bzl*-CHH-), 4.67 (ddd, 1H, 2nd Asp-$^{\alpha}$CH), 4.60 (ddd, 1H, 1st Phe-$^{\alpha}$CH), 4.38 (m, 2nd part of 1st and 2nd AB-system, 3H, 2×*Bzl*-CHH- and 2nd Phe-$^{\alpha}$CH), 4.28 (ddd, 1H, 1st Ala-$^{\alpha}$CH), 4.24-4.04 (m, 7H, 2nd Ala-$^{\alpha}$CH, 1st and 2nd Cys-$^{\alpha}$CH, 1st and 2nd -O-CH_2-), 3.73-3.58 (6s, 18H, 2×1st and 2nd 8,12-$COOCH_3$, 1st and 2nd Asp-O-CH_3), 3.29 (dd, 2H, 1st and 2nd Phe-$^{\beta}$CH(1)), 3.12 (qd, 1H, 1st 3,-H), 3.04-2.40 (m, 31H, 2×1st and 2nd 8,12-CH_2-, 1st and 2nd Cys-$^{\beta}CH_2$ 2nd 3,-H, 1st and 2nd 3-H, 1st and 2nd Phe-$^{\beta}$CH(2), 1st and 2nd Asp-$^{\beta}CH_2$, 1st and 2nd 2-H, 2×1st and 2nd 8,12-CH_2-COO-), 2.30

(m, 4H, 1st and 2nd 18-CH_2-CH_3) 2.16-2.03 (4s, 12H, 1st and 2nd 17-CH_3, 1st and 2nd 13-CH_3), 2.02, 1.80 (2s, 6H, 1st 7-CH_3 and 2nd 7-CH_3), 1.47 (d, 6H, 1st and 2nd 3′-CH_3), 1.30 (d, 3H, $J(^{\beta}CH_3,^{\alpha}CH) = 5.1$ Hz, 1st Ala-$^{\beta}$-CH_3), 1.22 (d, 3H, J(2-CH_3,2-H) = 5.4 Hz, 1st 2-CH_3), 1.18 (d, 3H, J(2-CH_3,2-H) = 5.4 Hz, 2nd 2-CH_3), 1.13 (d, 3H, $J(^{\beta}CH_3,^{\alpha}CH) = 5.1$ Hz, 2nd Ala-$^{\beta}CH_3$), 1.06 (t, 6H, 1st and 2nd 18-CH_2-CH_3), 0.95 (m, 4H, 1st and 2ndCH_2-Si-), 0.28 (2s, 18H, 1st and 2nd -Si$(CH_3)_3$) ppm; ^{1}H NMR (360 MHz, C_6D_5-CD_3, $T = 358$ K (above T_c)): $\delta = 7.40$-6.80 (m, 14H, Asp-NH, Phe-phenyl-H, *Bzl*-H Cys-NH, 10-H and Ala-NH), 6.73 (broad d, 1H, Phe-NH), 5.83 (s, 1H, 15-H), 5.49 (s, 1H, 5-H), 4.90 (broad, dd, 2H, *Bzl*-CH_2), 4.81 (m, 1H, Asp-$^{\alpha}$CH), 4.63 (m, 1H, Phe-$^{\alpha}$CH), 4.40 (m, 1H, Cys-$^{\alpha}$CH), 4.30 (m, 1H, Ala-$^{\alpha}$CH), 4.15 (m, 2H, -O-CH_2-) 3.40, 3.39 (2s, 9H, 2×8,12-$COOCH_3$, Asp-$COOCH_3$), 3.25-2.65 (m, 12H, Phe-$^{\beta}CH_2$3′-H, Cys-$^{\beta}CH_2$ 2-H, Asp-$^{\beta}CH_2$, 2×8,12-CH_2-), 2.59 (broad s, 1H, 3-H), 2.51 (m, 4H, 2×8,12-CH_2-COO-) 2.32 (m, 3H, 18-CH_2-CH_3) 2.01 (s, 3H, 7-CH_3), 1.94 (s, 3H, 13-CH_3), 1.88 (s, 3H, 17-CH_3), 1.25 (d, 3H, 2-CH_3), 1.18, (m, 6H, 18-CH_2-CH3, Ala-$^{\beta}CH_3$), 1.06 (d, 3H, J(3-CH_3,3′-H) = 7.0 Hz, 3′-CH_3), 0.90 (m, 2H, -CH_2-Si-), −.043 (s, 9H -Si$(CH_3)_3$ ppm; IR ($CHCl_3$): $\tilde{\nu} = 3425$, 3330, 3027, 2875, 1734, 1669,1636, 1595 cm^{-1}; UV/Vis (298 K, λ_{max} (ε)): $CHCl_3$: 592 (15100), 352 (34200), 275 (20700) nm; $CHCl_3$ (*TFA*): 632 (38900), 352 (25600), 331 (26300), 276 (13400), 260 (13000) nm; $CHCl_3$ (Zn^{2+}): 656 (17500), 382 (24700), 354 (25400) nm; $CHCl_3$ (*TBD*), guanidine base): 762 (20600), 408 (25500), 364 (29500), 314 (18960) nm; CD (298 K, λ_{max} ($\Delta\varepsilon$)): $CHCl_3$: 585 (−77), 351 (133), 277 (−47) nm; $CHCl_3$ (*TFA*): 623 (−30), 353(46), 275 (−19) nm; $CHCl_3$ (*TBD*): 760 (−77), 413 (28), 379 (26), 354 (24) nm.

(4Z,9Z,15Z,2R,3R,3′R,CysR,AlaS,PheS,AspS)-3-(1-(N-Benzyloxycarbonyl-cysteinyl-alanyl-phenylalanyl-α-mehyl-aspartyl-S-yl)-ethyl)-18-ethyl-2,3-dihydro-8,12-bis-(2-methoxycarbonylethyl)-2,7,13,17-tetramethyl-23H-bilin-1,19-(21H,24H)-dione
(**8**; $C_{63}H_{76}N_8O_{15}S$)

TBAF (4 mg, 12.6 μmol) was added to a solution of **7** (5 mg, 3.8 μmol) in 2 cm^3 of *THF*. The reaction mixture was stirred under Ar for 40 min, diluted with 50 cm^3 of CH_2Cl_2, washed with 40 cm^3 of 0.2 *M* aqueous $NaHCO_3$, 40 cm^3 0.1N aqueous HCl, 3×100 cm^3 H_2O and dried over Na_2SO_4. After evaporation of the solvent the residue was purified by column chromatography (silica gel, CH_2Cl_2/MeOH = 8/1) to afford **8** (4.2 mg, 90%) as a blue solid.

M.p.: 152°C $R_f = 0.38$ (silica gel, CH_2Cl_2/MeOH = 10/1); ^{1}H NMR (500 MHz, *DMSO*-d_6): $\delta = 9.79$ (broad s, 1H, chromophore-NH), 8.36 (d, 1H, Asp-NH), 8.14 (d, 1H, Ala-NH), 7.91 (d, 1H, J(NH,$^{\alpha}$CH) = 7.4 Hz, Phe-NH), 7.54 (d, 1H, J(NH,$^{\alpha}$CH) = 8.4 Hz, Cys-NH), 7.37-7.10 (m, 10H, Phe-phenyl-H, *Bzl*-H), 6.78 (s, 1H, 10-H), 5.96 (s, 1H, 15-H), 5.76 (s, 1H, 5-H), 4.96 (s, 2H, *Bzl*-CH_2-), 4.59 (ddd, 1H, Asp-$^{\alpha}$CH), 4.49 (ddd, 1H, Phe-$^{\alpha}$CH), 4.20 (ddd, 2H, Ala-$^{\alpha}$CH and Cys-$^{\alpha}$CH), 3.57 (s, 3H, Asp-O-CH_3), 3.55 (s, 6H, 2×8,12-$COOCH_3$), 3.34 (m, 1H, 3′-H), 3.10-2.35 (m, 15H, Phe-$^{\beta}CH_2$, 3-H, Cys-$^{\beta}$ CH_2, 8,12-CH_2-, 8,12-CH_2-COO-, Asp-$^{\beta}CH_2$), 2.28 (m, 1H, 2-H), 2.16 (q, 2H, 18-CH_2-CH_3), 2.06 (s, 3H, 17-CH_3), 2.05 (s, 3H, 13-CH_3), 1.94 (s, 3H, 7-CH_3), 1.28 (d, 3H, J(3′-CH_3,3′-H) = 6.6 Hz, 3′CH_3), 1.10 (m, 6H, 2-CH_3 and Ala-$^{\beta}CH_3$), 0.83 (t, 3H, J(18-CH_2-CH_3,18-CH_2-CH_3) = 6.7 Hz, 18-CH_2-CH_3) ppm; ROESY (500 MHz, *DMSO*-d_6, 298 K): Asp-NH↔Phe-$^{\alpha}$CH, Ala-NH↔(Cys-$^{\alpha}$CH, Ala-$^{\beta}CH_3$), Phe-NH↔(Ala-$^{\alpha}$CH, Phe-$^{\beta}$CH), *Bzl*-2,6H↔*Cbz*-CH_2-, 10-H↔(8,12-CH_2-, 8,12-CH_2-COO-), 15-H↔(13-CH_3, 17-CH_3), 5-H↔(3′-H,3-H, 7-CH_3, Cys-$^{\alpha}$CH), Phe-$^{\alpha}$CH↔(Phe-phenyl-2,6H, Asp-NH), Cys-$^{\alpha}$CH↔(Ala-NH, 3′-H), 3′-H↔(5-H, Cys-$^{\alpha}$CH, weak (2-H)), Phe-$^{\beta}$CH↔Phe-phenyl-2,6H), 3-H↔(5-H,3′-CH_3, 2-CH_3), 12-CH_2-↔(10-H, 13-CH_3) 8-CH_2-↔(10-H, 7-CH_3), 2-H↔(3′-CH_3, weak (3′-H)); UV/Vis (298 K, λ_{max} (ε)): $CHCl_3$: 601 (13600), 357 (28500), 276 (16600) nm; $CHCl_3$ (*TFA*): 637 (35700), 333 (27600), 278 (10400) nm; $CHCl_3$ (HOAc): 612 (17000), 356 (28300), 276 (14900) nm; $CHCl_3$ (Zn^{2+}: 641 (13800), 378 (21900), 355 (21400), 283 (13200) nm; CD (298 K, λ_{max} ($\Delta\varepsilon$)): $CHCl_3$: 578 (−54), 353 (90), 277 (−38) nm; $CHCl_3$ (*TFA*): 631 (9), 389 (−2), 345 (3), 283 (−10) nm; $CHCl_3$ (excess of HOAc): 587 (−38), 353 (57), 277 (−27) nm; MS (ESIp, *TFA*): *m*/*z* (%): 1217 $[M+H]^+$ (45), 609 (38), 242 (100).

Acknowledgements

We thank Dr. *W. Ahrer* for mass spectroscopic measurements. This work was supported by the Austrian Science Foundation (P9166-CHE).

References

[1] Stanek M, Grubmayr K (1998) Chem Eur J **4**: 1653
[2] Huber R (1989) Angew Chem **101**: 849; Angew Chem Int Ed Engl **28**: 848
[3] Glazer AN (1989) J Biol Chem **264**: 1
[4] Gärtner W (1997) Biol unserer Zeit **27**: 235
[5] Sineshchekov VA (1995) Biochim Biophys Acta **1228**: 125
[6] Duerring M, Schmidt, Huber R (1991) J Mol Biol **217**: 577
[7] Betz M (1997) Biol Chem **378**: 167
[8] Brejc M, Ficner R, Huber R, Steinbacher S (1995) J Mol Biol **249**: 424
[9] Duerring M, Huber R, Bode W (1990) J Mol Biol **211**: 633
[10] Ficner R, Lobeck K, Schmidt G, Huber R (1992) J Mol Biol **228**: 935
[11] Chang WR, Jiang T, Wan ZL, Zhang JP, Yang ZX, Liang DC (1996) J Mol Biol **262**: 721
[12] Schneider S, Geiselhart P, Baumann F, Siebzehnrübl S, Fischer R, Scheer H (1998) Photosynthetic Light-Harvesting Systems. Walter de Gruyter, Berlin, p 469
[13] Falk H (1989) The Chemistry of Linear Oligopyrroles and Bile Pigments. Springer, Vienna New York, p 190
[14] Berns S, MacColl R (1989) Chem Rev **89**: 807
[15] Scharnagl C, Schneider S (1991) J Photochem Photobiol B Biol **8**: 129
[16] Gillbro T, Sharkov AV, Kryukov IV, Khoroshilov EV, Fischer R, Scheer H (1993) Biochim Biophys Acta **1140**: 321
[17] Debreczeny MP, Sauer K, Zhou J, Bryant DA (1995) J Phys Chem **99**: 8412
[18] Debreczeny MP, Sauer K, Zhou J, Bryant DA (1995) J Phys Chem **99**: 8420
[19] Falk H, Müller N, Wansch S (1985) Monatsh Chem **116**: 1087
[20] Zrunek U, Falk H (1983) Monatsh Chem **114**: 983
[21] Zrunek U, Wolschan P, Falk H (1984) Monatsh Chem **115**: 243
[22] Krois D (1991) Monatsh Chem **122**: 495
[23] Stanek M, Grubmayr K (1998) Chem Eur J **4**: 1660
[24] Apt KE, Collier JL, Grossman AR (1995) J Mol Biol **248**: 79
[25] Chou PY, Fasman GD (1974) Biochemistry **13**: 211
[26] Richardson JS, Richardson DC (1988) Science **240**: 1648
[27] Carpino L, Beyermann M, Wenschuh H, Bienert M (1996) Acc Chem Res **29**: 268
[28] Jakube HD (1996) Peptide – Chemie und Biologie. Spektrum Akademischer Verlag, Heidelberg Berlin Oxford, p 151
[29] Carpino LA (1993) J Am Chem Soc **115**: 4397
[30] Carpino LA, El-Faham A, Albericio F (1994) Tetrahedron Lett **35**: 2279
[31] Sieber P (1977) Helv Chim Acta **60**: 2711
[32] Carpino LA, Han GY (1972) J Org Chem **37**: 3404

Received March 15, 2000. Accepted March 21, 2000