Efficient Syntheses of a Flavin and an 8-Hydroxy-5-deazaflavin Amino Acid and Their Incorporation into Oligopeptides

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We report a convenient synthesis for the cofactor amino acids (*S*)-**3** and (*S*)-**4** in which the C_5-ribose chain of the original riboflavin and ribo-5-deazaflavin cofactors is replaced by a C_5-amino acid side chain. Both cofactor amino acids are available in enantiomerically pure form in gram quantities and can be incorporated into oligopeptides using a standard Fmoc-based solid-phase peptide synthesis protocol. The benzyl-protecting group of the 8-hydroxy-5-deazaflavin can be cleaved by hydrogenolysis directly on the peptide. This allows the investigation of the properties of the peptide bound redox active OH- and the deprotonated O^--form of the deazaflavin. Due to the electron- and energy-transfer properties of both cofactors, applications of both amino acid in the preparation of peptide- and protein-based biosensors, of catalytically active peptides, or as chemical rulers for distance measurements in biopolymers based on the fluorescence resonance energy-transfer technology can be envisaged.

Introduction

The variety of organic reactions that can be catalyzed by proteins is restricted by the side-chain functionalities of the 21 encoded amino acids (including selenocysteine). In several enzymes this limitation is overcome by the incorporation of cofactors, which are bound to the protein in special cofactor-binding pockets.[1] The protein provides a suitably shaped cofactor binding site and delivers the appropriate side-chain functionalities required for the catalytic process. The activity of the cofactor is further modulated by the protein through the control of (1) the polarity of the cofactor binding pocket, (2) H-bonds or salt bridges to specific H-bond donor or acceptor groups of the cofactor,[2] and (3) the protonation or deprotonation of the cofactors in various redox states.[3] The desire to learn how cofactor-dependent enzymes modulate cofactor activity and the wish to prepare functionalized, and possibly catalytically active, oligopeptides and proteins as artificial enzymes[4−7] and biosensors[8−10] have motivated the preparation of cofactor−peptide chimeras.[11−13] Here, the incorporation of *cofactor amino acids* into oligopeptides using convenient solid phase peptide synthesis (SPPS) methodology was particularly successful and has recently enabled the synthesis of pyridoxalphosphate−, thiamine−, and nicotinamide−cofactor-containing oligopeptides.[13]

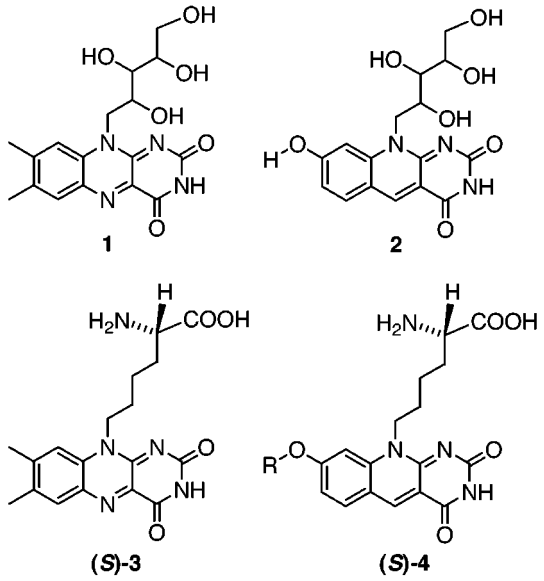

Figure 1. Depiction of the riboflavin **1** and the ribo-5-deazaflavin **2** cofactors and of the two C_5-chain flavin (*S*)-**3** and 5-deazaflavin (*S*)-**4** amino acid. R = $CH_2C_6H_6$.

Riboflavin (**1**) (Figure 1) is one of the most versatile cofactors used in nature.[14−16] It participates in reactions such as amino acid deamination, activation of molecular oxygen for oxidation reactions, one- and two-electron-transfer reactions, and in a photoinduced electron-

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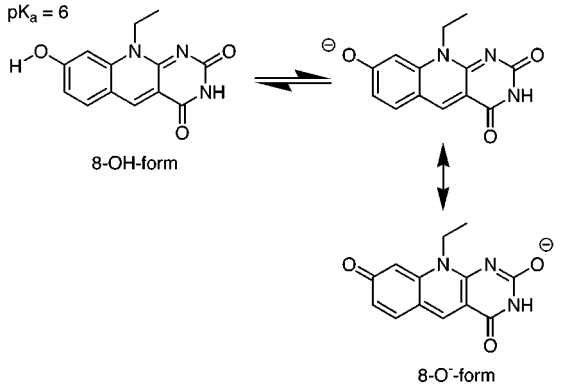


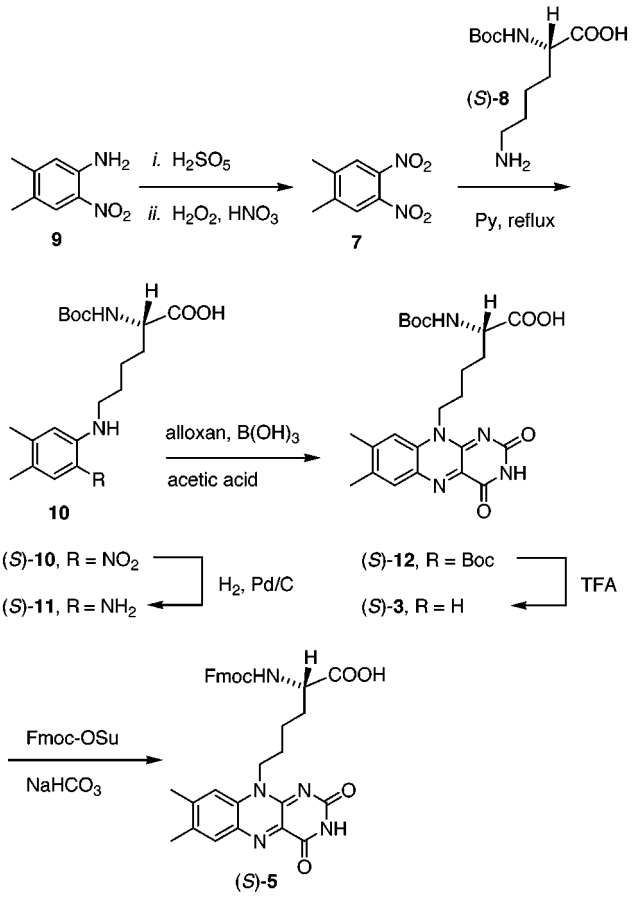
Figure 2. Representation of the 5-deazaflavin cofactor in its redox competent "protonated" (8-OH-form) and its rather redox incompetent deprotonated form (8-O$^-$-form).

transfer process required for the repair of pyrimidine dimer DNA lesions by the enzyme DNA-photolyase.[17] The structurally related ribo-5-deazaflavin (**2**) (Figure 1) functions in its "protonated" "8-OH-form" (Figure 2) as a nicotinamide in "flavin clothing",[18] as a low potential hydride-transfer agent involved in methane metabolism. The deprotonated "8-O$^-$-cofactor form" (p$K_a \approx 6$) participates as a light-gathering antenna unit in the repair of pyrimidine dimer DNA lesions.[19,20] For the preparation of defined redox active and potentially catalytically active oligopeptides, it would therefore be highly desirable to possess both cofactors as cofactor amino acid building blocks, amenable to SPPS. Furthermore, since both the flavin and the deazaflavin chromophore are known to be highly fluorescent and undergo light-induced electron- and energy-transfer reactions,[21] applications of flavin and deazaflavin amino acids in the synthesis of peptide- and protein-based biosensor materials[8,22,23] or as probes for distance measurements in biopolymers based on the fluoresecence resonance energy-transfer methodology can be envisioned.

Results and Discussion

In this paper, we report a convenient synthetic strategy for the preparation of the flavin[24] and the 5-deazaflavin amino acids (*S*)-**3** and (*S*)-**4** in which the C_5-ribose chain of the original riboflavin and ribo-5-deazaflavin cofactors is replaced by a C_5-amino acid side chain, which is derived retrosynthetically from a lysine building block. Both cofactor amino acids are available in enantiomerically pure form in gram quantities as required for efficient SPPS. To study both, the redox active OH- and the deprotonated O$^-$-forms of the deazaflavin, we prepared the 8-*O*-benzyl-protected 5-deazaflavin amino acid (*S*)-**4** (R = Bn), which mimics the deazaflavin cofactor in its "protonated" status. Deprotection of the 8-*O*-benzyl group, using catalytic hydrogenation and raising of the pH (pH > 7), converts the deazaflavin into its deprotonated form.

Scheme 1. Synthesis of the Fmoc-Protected Flavin Amino Acid (*S*)-5



The synthesis of the (*S*)-2-amino-N^{α}-(9-fluorenylmethyloxycarbonyl)-6-(7′,8′-dimethylisoalloxazin-10′-yl)hexanoic acid [(*S*)-**5**] and of the (*S*)-2-amino-N^{α}-(9-fluorenylmethyloxycarbonyl)-6-(8′-*O*-benzyl-5′-carbaisoalloxazin-10′-yl)hexanoic acid [(*S*)-**6**] are outlined in Schemes 1 and 2. The key step in the preparation of the Fmoc-protected flavin amino acid (*S*)-**5** involves an ipso-substitution reaction between 4,5-dimethyl-1,2-dinitrobenzene (**7**) and the N^{α}-Boc-protected (*S*)-lysine derivative (*S*)-**8**. The 1,2-dinitrobenzene starting material **7** was prepared from 4,5-dimethyl-2-nitroaniline (**9**) using a known two-step oxidation protocol, first involving oxidation of **9** to the nitroso-nitro compound with Caros' acid and then further oxidation of this intermediate to the dinitro compound **7** with hydrogen peroxide and catalytic nitric acid.[25,26] The ipso-substitution was found to proceed without racemization of the reaction product (*S*)-**10** in an optimal fashion either in a solvent mixture of *n*-butanol, water, and ethanol at 80 °C with potassium acetate as the base or in pure pyridine at 100 °C. The intensively red-colored reaction product (*S*)-**10** was readily separated from excess dinitro compound **7** even on larger scale by filtration through a silica gel plug eluting first with chloroform/TEA to remove **7** and then with chloroform/methanol/

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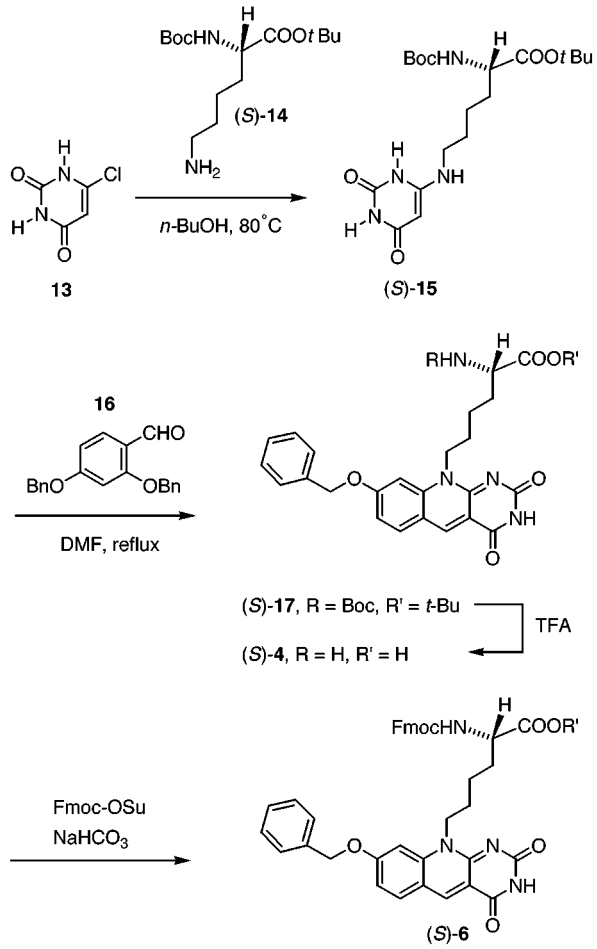
Scheme 2. Synthesis of the Fmoc-Protected Deazaflavin Amino Acid (*S*)-6



acetic acid (5:1:1%) to obtain the desired product (*S*)-**10**. Hydrogenolytic reduction of the nitro group in (*S*)-**10** and subsequent filtration of the reaction product (*S*)-**11** through Celite into a suspension of alloxan and boric acid in acetic acid, following the general flavin synthesis protocol of R. Kuhn and co-workers[27,28] yielded the N^{α}-Boc-protected flavin amino acid (*S*)-**12**. Cleavage of the Boc-protection group with trifluoroacetic acid afforded the unprotected flavin amino acid (*S*)-**3** which was reacted with Fmoc-OSu in sodium hydrogen carbonate solution to yield the desired N^{α}-Fmoc-protected flavin amino acid (*S*)-**5** in 16% overall yield as an intensively yellow-colored powder, ready for the incorporation into peptides using SPPS.

For the preparation of the deazaflavin amino acid (*S*)-**6**, the 6-chloro uracil **13** was first reacted with the N^{α}-Boc-protected (*S*)-lysine-*tert*-butyl ester building block (*S*)-**14**[29,30] in *n*-butanol at 80 °C to yield the uracil-lysine compound (*S*)-**15**. Protection of the amino and carboxylic acid functionalities in (*S*)-**14** as the *tert*-butyl carbamate and the *tert*-butyl ester, respectively, was required to increase the solubility and allow purification of the lysine−uracil reaction product (*S*)-**15** by chromatography. Subsequent reaction of (*S*)-**15** with the bisbenzyl-protected 2,4-dihydroxybenzaldehyde **16**, following the general deazaflavin synthesis protocol of F. Yoneda and co-workers[31] afforded the N^{α}-Boc-protected deazaflavin amino acid (*S*)-**17**. This was treated with trifluoroacetic acid to cleave both *tert*-butyl-containing protection groups to afford (*S*)-**4**. Reaction of the unprotected deazaflavin amino acid (*S*)-**4** with Fmoc-OSu and sodium hydrogen carbonate then yielded the N^{α}-Fmoc-protected deazaflavin amino acid (*S*)-**6**. The final Fmoc-protected deazaflavin amino acid (*S*)-**6** was obtained as a light yellow powder in 15% overall yield, ready for its incorporation into oligopeptides using SPPS. Despite the only moderate yields obtained in the ipso-substitution reaction between **7** and (*S*)-**8**, and in the condensation reaction of (*S*)-**11** with alloxan and of (*S*)-**15** with the benzaldehyde derivative **16** to give the isoalloxazine and 5-carbaisoalloxazine heterocycles (*S*)-**12** and (*S*)-**17**, both cofactor amino acids (*S*)-**5** and (*S*)-**6** are readily available in gram quantities using the above-described procedures.

To determine the enantiomeric purity of the cofactor amino acids (*S*)-**3** and (*S*)-**4**, we prepared the corresponding (*R*)-configured compounds (*R*)-**3** and (*R*)-**4** using the same procedures and reaction conditions but the (*R*)-configured lysine starting materials (*R*)-**8** and (*R*)-**14**. The racemates *(rac)*-**3** and *(rac)*-**4** were obtained by mixing the corresponding enantiomers. Separation of the enantiomers was accomplished by thin layer, ligand exchange chromatography on reverse phase CHIRALPLATES.[32] The solvent mixture acetonitrile/water/methanol (4:1:1) allowed the rapid (20 min) optical resolution of the racemates *(rac)*-**18**, *(rac)*-**3**, and *(rac)*-**4**. Separation of the racemate of the deprotected compound derived from *(rac)*-**15** was not possible using the CHIRALPLATE technology due to low solubility. A dilution experiment, in which decreasing amounts of the (*R*)-isomers (*R*)-**18**, (*R*)-**3**, and (*R*)-**4** were added to the corresponding (*S*)-forms and subsequent scanning of the obtained TLC plates established that amounts of the (*R*)-isomers down to 2−4% are detectable. Since the final compounds derived from the *(S)*-configured starting material (*S*)-**3** and (*S*)-**4** yield only one spot on the CHIRALPLATES, as depicted in Figure 3 for the amino acids (*R*/*S*)-**18** and (*R*/*S*)-**3**, we conclude that the final synthetic cofactor amino acids (*S*)-**5** and (*S*)-**6** possess an enantiomeric purity of ee > 95%.

Experiments aimed at finding suitable conditions for the incorporation of the flavin and the deazaflavin amino acids (*S*)-**5** and (*S*)-**6** into oligopeptides by SPPS revealed that both cofactor amino acids are fully amendable to a standard Fmoc-based SPPS protocol.[33] Activation of the cofactor amino acids was performed with a mixture of HOBT and TBTU for 5 min prior to their coupling. The coupling reaction was performed either in NMP or in DMF using a Rink resin. Brief capping after each coupling was performed with acetic anhydride and DIEA. Deprotection of the Fmoc-group was achieved with 20% piperidine in DMF. This causes no decomposition of the otherwise nucleophile-sensitive isoalloxazin heterocycle. Cleavage of the side-chain protection groups and of the

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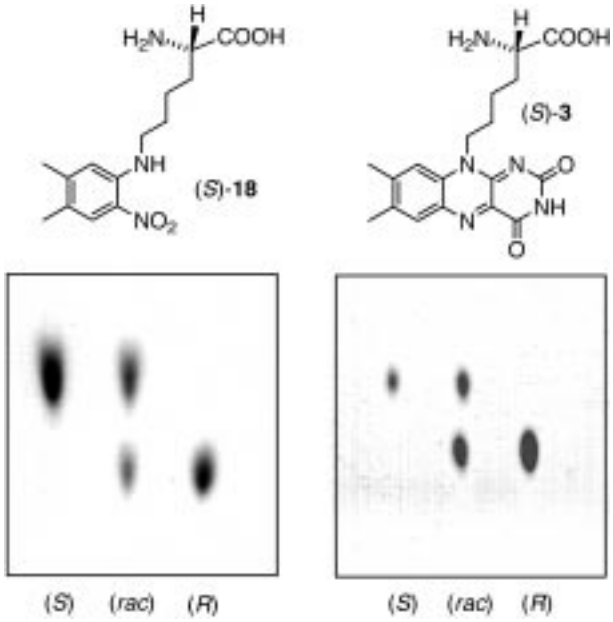

Figure 3. Depiction of the optical resolution of the racemates (*rac*)-**18** and (*rac*)-**3** with CHIRALPLATES[32] (solvent system of acetonitrile, water, methanol (4:1:1)).

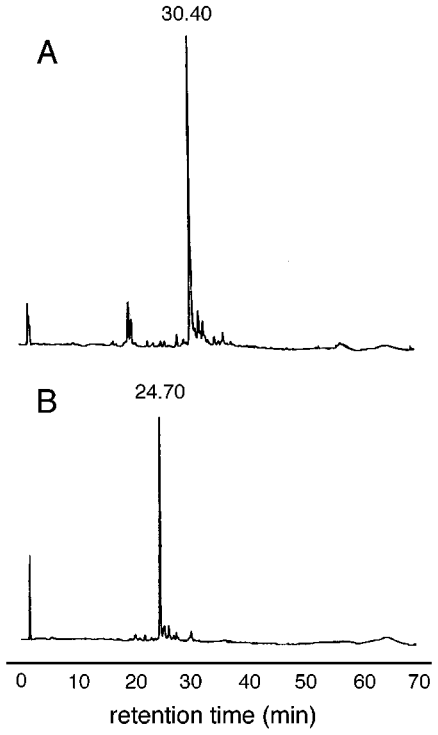

Figure 4. HPLC traces of the crude peptides **19** (A) and **20** (B) obtained directly after the acid-mediated cleavage from the solid support. Reverse phase HPLC. Gradient 100% A to 80% B in 70 min (A, water with 0.1% TFA; B, acetonitrile).

peptide from the resin was achieved with 95% trifluoroacetic acid, 2.5% triisopropylsilane, and 2.5% water over 5 h. The crude peptide solution was evaporated in vacuo and precipitated with diethyl ether. Figure 4 shows the RP-HPLC traces obtained from the crude flavin- and deazaflavin-containing peptides **19** and **20** (Table 1) directly after acid-mediated cleavage from the solid support. The cofactor peptides **19** and **20** are the main reaction products and are obtained with a purity comparable to the reference Ala-containing peptide **21** (HPLC trace not shown). The successful preparation of the 24- and 27-mer oligopeptides **23** and **22** demonstrates that even a large number of coupling cycles can be performed after the incorporation of the cofactor amino acids without significant cofactor destruction. The purification of the cofactor peptides was finally performed by preparative HPLC with a water (0.1% TFA)/acetonitrile gradient. Here the strong absorption of both heterocycles at 450 nm (flavin) and 400 nm (deazaflavin) allowed their easy detection and facilitated the HPLC purification procedures.

To cleave the 8-*O*-benzyl-protection group of the deazaflavin and to convert the deazaflavin into its deprotonated form, deazaflavin-containing peptides such as **24** were dissolved in an acetic acid/water mixture (1:1) and a very small amount of $Pd/BaSO_4$ catalyst was added.[21] This solution was gently stirred in an H_2 atmosphere for approximately 6 h. During this time, the fluorescence of the sample (excitation at 366 nm) was carefully monitored. It proved to be essential to avoid complete reduction of the deazaflavin chromophore, which is accompanied by a diminishment of the characteristic blue fluorescence. After approximately 80% conversion, isolation of the debenzylated peptide **25** was achieved by preparative HPLC.

Lyophilization of the purified peptides yielded all cofactor peptides in the form of intensively orange or yellow powders which were characterized by HPLC, electrospray or MALDI-TOF mass spectrometry, and amino acid sequencing.

The UV and fluorescence spectra of the purified peptides **19** and **23**−**25** in water at pH = 8 are depicted as typical examples in Figure 5. They prove the presence of the cofactors, all stably incorporated into the oligopeptides. The benzylated deazaflavin-containing peptides (such as **24**) feature the expected absorption maxima around 400 nm for the OH-form deazaflavin. The flavin peptides **19** and **23** exhibit absorption maxima at 370 and 450 nm. The intense fluorescence at 430 nm (**24**) and 520 nm (**23**) shows the successful incorporation of the intact heterocycles. UV/vis and fluorescence spectra underline the successful debenzylation of the deazaflavin cofactor inside the oligopeptide. The measured UV/vis and fluorescence spectrum of the peptide **25**, containing the debenzylated deazaflavin, features under acidic conditions the absorption and fluorescence characteristics of the benzylated deazaflavin unit. Addition of triethylamine, or measuring in water, buffered at pH 8, however, caused deprotonation of the 8-OH group ($pK_a = 6$) as evidenced by the increased absorption, the shift of the absorption maximum from approximately 400 to 420 nm and by the fluorescence maximum which appears now at 470 nm, as expected for the deprotonated 8-hydroxy-5-carbaisoalloxazine chromophore. Both values are in full agreement with the spectroscopic data of the *A. nidulans* DNA-photolyase, which contains the F420 cofactor as a light-gathering antenna chromophore in its deprotonated form. Investigation of the fluorecence properties of the flavin amino acid incorporated into the tyrosine-rich peptide **19** shows that the flavin fluorescence is strongly reduced (Figure 5B). This experiment confirms that the flavin environment critically influences the spectroscopic properties of the cofactor and supports

Table 1. Sequence of the Prepared Cofactor Peptide Chimeras and Their Calculated and Determined Molecular Weights

no.	sequence	calcd [M^+]	obsd [M^+] ($M^+ + Na$)
21	AYA-A-EYYLE-OH	1092	(1118)[a]
19	AYA-Fl-EYYLE-OH	1375	1375[b] (1397)[b]
20	AYA-dFl(OBn)-EYYLE-OH	1452	1451[b] (1474)[b]
24	AQDKA-dFl(OBn)-SKA-NH_2	1248	1247[b]
25	AQDKA-dFl-SKA-NH_2	1158	1157[b]
22	PAAL-Fl-RARNTEAARRSRARKLQRLKEC-NH_2	3374	3375[a]
23	Ac-ADRRKAATERERRR-Fl-SKVNEAGGC-NH_2	3012	3011[a]

[a] Maldi-TOF mass spectrometry. [b] Electrospray mass spectrometry. Fl = Flavin, dFl = deazaflavin, dFl(OBn) = 8-*O*-benzyl-deazaflavin. A: Alanine. C: Cysteine. D: Aspartic acid. E: Glutamic acid. G: Glycine. K: Lysine. L: Leucine. N: Asparagine. P: Proline. Q: Glutamine. R: Arginine. S: Serine. T: Threonine. V: Valine. Y: Tyrosine.

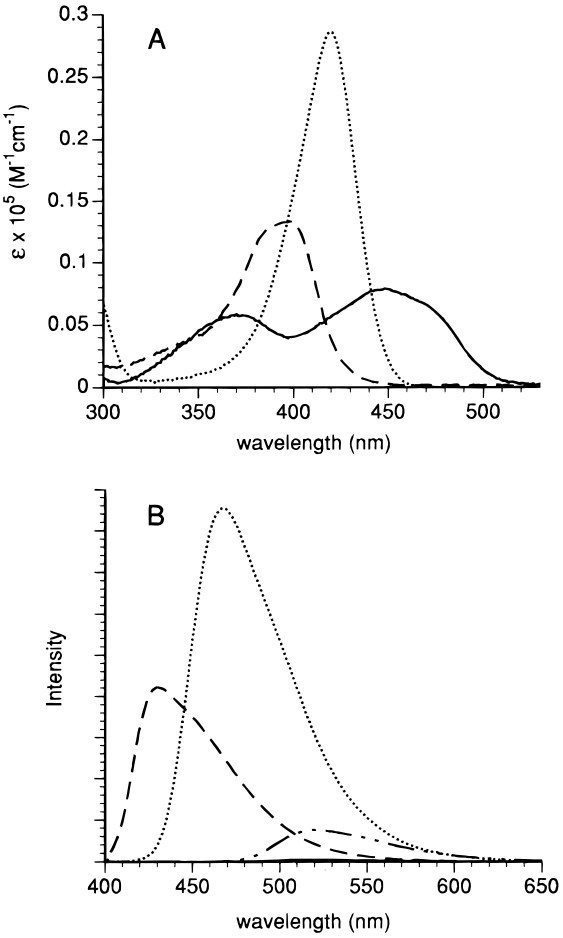

Figure 5. UV/Vis spectra (A) and fluorescence spectra (B) of the flavin and deazaflavin containing peptides measured at 10^{-5} M in water (0.05 M Tris·HCl, pH = 8). A: (—) **19** and **23**. (– – –) **24**. (···) **25**. B: (—) **19**. (—··) **23**. (– – –) **24**. (···) **25**.

the observation of McCormick and co-workers of reduced flavin fluorescence in a tyrosine or tryptophan environment.[34]

Conclusions

In this paper, we describe an efficient and convenient synthesis for the flavin and the deazaflavin amino acid (*S*)-**3** and (*S*)-**4**. Both cofactor amino acids can be incorporated into oligopeptides using a rather standard Fmoc-based solid-phase peptide synthesis protocol. The incorporated cofactors show all of the expected spectroscopic properties of a flavin and a deazaflavin which illustrates their stable incorporation. Debenzylation of the benzyl-protected 8-OH-deazaflavin group was achieved using catalytic hydrogenolysis on the peptide. Although this deprotection requires special care, it allows the study of both forms (8-OH and 8-O^-) of the deazaflavin cofactor using the same building block for SPPS. We communicated recently that flavin-containing oligopeptides, which possess the sequence of DNA-binding transcription factors, are able to repair thymine dimer DNA-lesions in a DNA single strand.[24] We believe the presented procedures now enable the preparation of flavin and deazaflavin peptides as artificial DNA-repair enzymes and of oligopeptides with novel energy- and electron-transfer properties.[35] Due to the ability of deazaflavins to transfer excitation energy to the flavin,[21] both cofactor can also be used as new tools for fluorescence energy-transfer experiments in biopolymers.

Experimental Section

General. Reagents and solvents were purchased reagent grade and used without further purification. Anhydrous $MgSO_4$ was used as the drying agent after aqueous workup. Evaporation and concentration in vacuo was done at H_2O-aspirator pressure. Column chromatography: Silica gel-H or -S from Fluka. TLC: glass or aluminum sheets covered with silica gel 60 F_{254} from Merck; visualization by UV light. Melting points are uncorrected and measured in open capillary tubes. IR spectra (cm^{-1}) are measured as KBr pellets. Fluorescence spectra were measured on a Spex 1680, 0.22m double grating spectrometer, 450 Hg/Xe-lamp, in 1 cm quartz cuvettes at RT. For ^1H and ^{13}C NMR, solvent peaks (2.49 ppm for ^1H and 39.7 ppm for ^{13}C NMR, respectively) are used as reference. MS (*m*/*z*): FAB measured in a 3-nitrobenzyl alcohol matrix. α-Values were measured at 24 °C. HPLC was performed with a flow of 1 mL/min using a Nucleosil RP18 (240 mm × 4 mm, 100 Å/5 μm) column. For analytical oligopeptide HPLC, a linear gradient (H_2O (0.1%TFA) to acetonitrile) was used. Preparative HPLC was performed with a Nucleoprep RP C18 column (250 mm × 21 mm, 100 Å/10 μm). Solvent system: A = 0.1% TFA in H_2O, B = acetonitrile; A to B in 120 min.

4,5-Dimethyl-1,2-dinitrobenzene (7). Compound **9** (36 g, 0.22 mol) was dissolved in ice water (500 mL). Potassium persulfate (120 g) was added into concentrated sulfuric acid (200 mL), and the mixture was stirred until a clear solution was obtained. This solution was added into the suspension of **9** in ice water. The reaction mixture was stirred at RT for 20 h, and then another batch of potassium persulfate (60 g) in concentrated sulfuric acid (200 mL) was added until all starting material had disappeared. The intermediate nitroso product (41 g) was filtered off and dried in vacuo at 50 °C. The product was subsequently dissolved in concentrated acetic

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acid (550 mL) at 100 °C. Nitric acid (70 mL, 65%) was added. A mixture of H_2O_2 (35%, 500 mL) in concentrated acetic acid (200 mL) was then added to the hot solution over 1.5 h. The clear orange reaction solution was stirred for another 2 h at 100 °C. After complete reaction (TLC toluene/ethyl acetate 10:1), the reaction mixture was cooled to RT, and the solid product (21.4 g, 50%) **7** was filtered off and dried in vacuo at 50 °C. For elemental analysis, **7** was recrystallized from EtOH. For **7**: mp 111−112 °C; IR (KBr) 3100, 3044, 2922, 2367, 1761, 1583, 1551, 1528, 1483, 1444, 1379, 1338, 1256, 1237, 1146, 1024, 1002, 894, 883, 859, 807, 751, 660, 584, 548, 417; ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.44 (s, 6 H), 7.70 (s, 2 H); MS (EI) *m*/*z* (%) 196 (75) [M^+], 108, 91, 77, 65, 51, 39. Anal. Calcd for $C_8H_8N_2O_4$ (196.16): C, 48.93; H, 4.11; N, 14.28. Found C, 48.89; H, 3.90; N, 14.37.

(*S*)-2-Amino-*N*$^\alpha$-(*tert*-butyloxycarbonyl)-6-(4′,5′-dimethyl-2′-nitroanilin-*N*-yl)hexanoic Acid ((*S*)-10). Compound **7** (2.0 g, 10.2 mmol) and N$^\alpha$-(*tert*-butyloxycarbonyl)-(*S*)-lysine (*S*)-**8** (4.0 g, 16.2 mmol) were suspended in pyridine (250 mL), and the mixture was heated at reflux for 72 h. The dark red reaction solution was diluted with $CHCl_3$ (400 mL) and extracted three times with citric acid solution (10% in water). The organic phase was finally washed with water, dried with $MgSO_4$, and, after the addition of 50 g silica gel, evaporated to dryness. The resulting red powder was added on top of a silica gel column. Unreacted **7** was separated by eluting the column with $CHCl_3$ (1% TEA). The product (*S*)-**10** was separated with $CHCl_3$/MeOH (5:1, 1% acetic acid) as the eluent. After evaporation of the solvent in vacuo, (*S*)-**10** was obtained as a red oil that crystallized at 4 °C (3.0 g, 74%): $[\alpha]_D$ = 2.0 (*c* = 1.0% in DMSO); mp 74−76 °C; IR (KBr) 3378, 2978, 2922, 2867, 2356, 1700, 1678, 1633, 1572, 1506, 1406, 1367, 1306, 1239, 1161, 1056, 1022, 861, 761, 667; ^{1}H NMR (500 MHz) δ 1.35 (s, 9 H), 1.58 (m, 4H), 1.73 (m, 2H), 2.11 (s, 3H), 2.23 (s, 3H), 3.27 (m, 2H), 3.76 (br.s., 1H), 6.29 (br.s., 1H), 6.81 (s, 1H), 7.78 (s, 1H), 7.97 (t, *J* = 5.4 Hz, 1H); ^{13}C NMR (125.8 MHz) δ 17.9, 20.1, 22.7, 28.2, 28.3, 32.3, 42.3, 54.5, 77.4, 114.4, 123.9, 125.3, 128.5, 143.9, 147.5, 154.9, 166.9; MS (FAB$^-$) *m*/*z* (%) 394 (40) [M^- − H], 306 (100); HRMS calcd for $C_{19}H_{29}N_3O_6$ [M^- − H] = 394.1978, found 394.1982.

(*S*)-2-Amino-*N*$^\alpha$-(*tert*-butyloxycarbonyl)-6-(7′,8′-dimethylisoalloxazin-10′-yl)hexanoic Acid ((*S*)-12). (*S*)-2-Amino-N$^\alpha$-(*tert*-butyloxycarbonyl)-6-(4′,5′-dimethyl-2′-nitroanilin-*N*-yl-)hexanoic acid ((*S*)-**10**) (2.8 g, 7.1 mmol) was dissolved in acetic acid (100 mL) and stirred after the addition of cat. Pd/C (10%) for 24 h under an H_2 atmosphere. The colorless reaction solution was filtered through Celite, and subsequently alloxan monohydrate (3.0 g) and boric acid (4.0 g) were added. The mixture was stirred at RT in the dark for another 12 h. The solution was diluted with $CHCl_3$ (500 mL), and the organic phase was washed three times with water. The organic phase was separated, dried with $MgSO_4$, and evaporated in vacuo. Diethyl ether was added to the resulting oil in order to precipitate the product. Compound (*S*)-**12** was obtained as a yellow powder, which was dried in vacuo (2.0 g, 60%): $[\alpha]_D$ = −1.0 (*c* = 1.0% in DMSO); mp > 300 °C; IR (KBr) 3411, 2966, 2367, 1678, 1578, 1544, 1461, 1400, 1367, 1330, 1167, 1056, 1017, 861, 827, 811, 772, 739, 678, 605, 583, 499, 470, 452; ^{1}H NMR (400 MHz) δ 1.38 (s, 9 H), 1.48−1.85 (m, 6H), 2.39 (s, 3H), 2.51 (s, 3H), 3.88 (br.s., 1H), 4.55 (m, 2H), 7.04 (d, *J* = 8.1 Hz), 7.76 (s, 1H), 7.89 (s, 1H), 11.28 (s, 1H), 12.50 (br.s., 1H); ^{13}C NMR (100 MHz) δ 18.7, 20.7, 22.8, 25.9, 28.1, 30.5, 43.9, 53.2, 64.8, 77.9, 115.0, 130.6, 131.0, 133.7, 135.5, 137.0, 146.5, 149.9, 155.6, 159.9, 174.0; MS (FAB$^-$) *m*/*z* (%) 471.3 (45) [M^- − H]. Anal. Calcd for $C_{23}H_{29}N_5O_6$ + H_2O (471.51 + 18.02): C, 56.43; H, 6.39; N, 14.31. Found: C, 56.56; H, 6.50; N, 14.06.

(*S*)-2-Amino-6-(7′,8′-dimethylisoalloxazin-10′-yl)hexanoic Acid ((*S*)-3). (*S*)-2-Amino-N$^\alpha$-(*tert*-butyloxycarbonyl)-6-(7′,8′-dimethylisoalloxazine-10′-yl)hexanoic acid ((*S*)-**12**) (1.6 g, 3.4 mmol) was stirred in a mixture of $CHCl_3$/trifluoroacetic acid (1:1, 25 mL) for 1 h at RT under the exclusion of light. The reaction mixture was evaporated in vacuo, and diethyl ether was added to the resulting oil to precipitate the free amino acid (*S*)-**3**. The product (*S*)-**3** was filtered off and obtained as a yellow powder after reprecipitation from water/ethanol (1.2 g, 95%): $[\alpha]_D$ = 6.0 (*c* = 1.0% in DMSO); mp > 215 °C; IR (KBr) 3446, 2360, 1687, 1578, 1544, 1461, 1350, 1267, 1205, 1139, 839, 800, 722, 667, 606; ^{1}H NMR (400 MHz) δ 1.45−1.65 (m, 2 H), 1.70−1.78 (m, 2H), 1.80−1.95 (m, 2H), 2.38 (s, 3H.43 (s, 3H), 3.91 (t, *J* = 8 Hz, 1H), 4.58 (m, 2H), 7.78 (s, 1H), 7.88 (s, 1H), 8.15−8.40 (br.s., 1H), 11.30 (s, 1H); ^{13}C NMR (100 MHz) δ 18.7, 20.6, 21.5, 25.9, 29.6, 43.6, 51.9, 116.1, 130.6, 131.0, 133.8, 135.8, 137.1, 146.7, 150.1, 155.8, 159.9, 171.0; MS (FAB$^+$) *m*/*z* (%) 372.2 (30) [MH^+]; HRMS calcd for $C_{18}H_{21}N_5O_4$ [MH^+] 372.1672, found 372.1683.

(*S*)-2-Amino-*N*$^\alpha$-(9-fluorenylmethyloxycarbonyl)-6-(7′,8′-dimethylisoalloxazin-10′-yl)hexanoic Acid ((*S*)-5). (*S*)-2-Amino-6-(7′,8′-dimethylisoalloxazin-10′-yl)hexanoic acid ((*S*)-**3**) (1.0 g, 2.7 mmol) was dissolved in an aqueous sodium hydrogen carbonate solution (150 mL, 10%). The suspension was cooled to 0 °C, and Fmoc-OSu (1.1 g, 3.3 mmol) dissolved in DMF (15 mL) was added. The resulting thick slurry was shaken for another 30 min and diluted with water, and the aqueous phase was extracted with diethyl ether. The aqueous phase was collected, acidified with citric acid (pH = 4), and extracted three times with $CHCl_3$. The combined organic phases were dried with $MgSO_4$, filtered, and evaporated in vacuo. The product (*S*)-**5** was precipitated through the addition of diethyl ether and dried in vacuo. Compound (*S*)-**5** was obtained as a yellow powder (1.2 g, 75%): $[\alpha]_D^{20}$ = −13.1 (*c* = 1.0% in DMSO); mp > 90−95 °C (dec); IR (KBr) 3419, 2360, 1716, 1667, 1579, 1543, 1450, 1400, 1350, 1261, 1172, 741, 667; ^{1}H NMR (400 MHz) δ 1.48−1.60 (m, 2 H), 1.70−1.80 (m, 4H), 2.36 (s, 3H), 2.48 (s, 3H), 3.95−3.99 (m, 1H), 4.20−4.25 (m, 1H), 4.25−4.33 (m, 2H), 4.50−4.65 (m, 2H), 7.32 (dd, *J* = 14. Hz, 6.7 Hz, 2H), 7.41 (t, *J* = 7.4 Hz, 2H), 7.71 (m, 4H), 7.89 (m, 3H, Ar−H), 11.30 (s, 1H); ^{13}C NMR (100 MHz) δ 15.1, 18.7, 22.9, 25.9, 30.5, 43.9, 46.6, 53.6, 65.5, 115.9, 120.0, 125.2, 126.9, 127.5, 130.6, 130.9, 133.7, 135.6, 137.0, 140.6, 143.7, 146.5, 149.9, 155.6, 156.1, 159.8, 173.7; MS (FAB$^-$) *m*/*z* (%) 593.4 (100) [$M^{\bullet-}$]; HRMS calcd for $C_{33}H_{31}N_5O_6$ [$M^{\bullet-}$] 593.2274, found 593.2269. Anal. Calcd for $C_{33}H_{31}N_5O_6$ + 1.5 H_2O (620.68): C, 63.85; H, 5.52; N, 11.28. Found: C, 64.06; H, 5.61; N, 10.93.

(*S*)-2-Amino-*N*$^\alpha$-(*tert*-butyloxycarbonyl)-6-(6′-aminopyrimidine-2′,4′-dion-*N*-yl)hexanoic Acid *tert*-Butyl Ester ((*S*)-15). 6-Chloro uracil **13** (3.0 g, 20 mmol) and N$^\alpha$-(*tert*-butyloxycarbonyl)-(*S*)-lysine *tert*-butyl ester ((*S*)-**14**) (7.0 g, 23 mmol) were heated in *n*-butanol (50 mL) to 80 °C for 5 h. The reaction solution was evaporated in vacuo, and the residual material was dried in vacuo until a solid material was obtained. The product (*S*)-**15** was purified by chromatography on silica gel with $CHCl_3$/MeOH (20:1). Compound (*S*)-**15** was obtained as an off-white powder (4.1 g, 49%), which was used without further purification: $[\alpha]_D$ = −9.9 (1.0% in DMSO); mp = 173−176 °C; IR (KBr) 2878, 2922, 2356, 1713, 1615, 1517, 1450, 1389, 1368, 1289, 1244, 1155, 1044, 1022, 844, 781, 547; ^{1}H NMR (400 MHz) δ 1.25−1.50 (m, 22 H), 1.50−1.70 (m, 2H), 3.00 (m, 2H), 3.78 (m, 1H), 4.40 (s, 1H), 6.05 (m, 1H), 7.05 (d, *J* = 7.7 Hz, 1H), 9.83 (s, 1H), 10.15 (s, 1H); ^{13}C NMR (100 MHz) δ 22.8, 27.6, 27.7, 30.3, 41.0, 54.2, 72.4, 77.9, 80.1, 150.7, 153.9, 155.5, 164.1 (2C), 171.8; MS (FAB$^+$) *m*/*z* (%) 413.4 (95) [MH^+]. Anal. Calcd for $C_{19}H_{32}N_4O_6$ (412.49): C, 55.33; H, 7.82; N, 13.58. Found C, 55.32; H, 7.72; N, 13.46.

***(S)*-2-Amino-*N*$^\alpha$-(*tert*-butyloxycarbonyl)-6-(8′-*O*-benzyl-5′-carbaisoalloxazin-10′-yl)hexanoic Acid *tert*-Butyl Ester ((*S*)-17).** (*S*)-2-Amino-N$^\alpha$-(*tert*-butyloxycarbonyl)-6-(6′-aminopyrimidine-2′,4′-dion-*N*-yl)hexanoic acid *tert*-butyl ester ((*S*)-**15**) (1.5 g, 3.6 mmol) and 2,4-(dibenzyloxy)benzaldehyde (**16**) (2.3 g, 7.2 mmol) were dissolved in DMF (50 mL) and heated to 120 °C for 12 h. The solvent was evaporated in vacuo, and diethyl ether was added to the solid residual material yielding the product as a light yellow precipitate which was filtered off, washed with diethyl ether twice, and dried in vacuo. Compound (*S*)-**17** was obtained as a light yellow powder (900 mg, 41%): $[\alpha]_D^{20}$ = −10.4° (1.0% in DMSO); mp = 193−195 °C; IR (KBr) 3422, 3133, 2978, 2811, 2367, 1706, 1654, 1600, 1528, 1489, 1456, 1406, 1234, 1156, 1000, 850, 580, 444; ^{1}H NMR (400 MHz) δ 1.30−1.50 (m, 20 H), 1.55−1.70 (m, 4H), 3.80 (m, 1H), 4.65 (m, 2H), 5.43 (s, 2H),

7.10 (d, J = 7.7 Hz, 1H), 7.25 (m, 2H), 7.36 (m, 1H), 7.43 (t, J = 7.1 Hz, 2H), 7.52 (d, J = 7.1 Hz, 2H,), 8.11 (d, J = 9.5 Hz, 1H), 8.89 (s, 1H), 10.90 (s, 1H); ^{13}C NMR (100 MHz) δ 22.8, 24.5, 27.5, 27.9, 30.4, 43.9, 54.2, 70.1, 77.9, 80.1, 100.0, 111.9, 114.7, 115.8, 127.8, 128.1, 128.5, 133.7, 136.0, 141.0, 142.0, 145.2, 155.5, 156.5, 162.2, 164.0, 171.7; MS (FAB$^+$) *m*/*z* (%) 627 (30) [M^+ + Na], 605 (100) [M^+ + H], 449 (95). Anal. Calcd for $C_{33}H_{40}N_4O_7$ (604.70): C, 65.55; H, 6.67; N, 9.27. Found: C, 65.49; H, 6.72; N, 9.29.

***(S)*-2-Amino-6-(8′-*O*-benzyl-5′-carbaisoalloxazin-10′-yl)-hexanoic Acid ((*S*)-4).** *(S)*-2-Amino-N$^\alpha$-(*tert*-butyloxycarbonyl)-6-(8′-*O*-benzyl-5′-carbaisoalloxazin-10′-yl)hexanoic acid *tert*-butyl ester ((*S*)-**17**) (900 mg, 1.5 mmol) was stirred in trifluoroacetic acid (15 mL) and water (0.5 mL) for 3 h at RT under the exclusion of light. The reaction mixture was evaporated in vacuo, and diethyl ether was added to the residual oil to precipitate the trifluoroacetic acid salt of the product (*S*)-**4**·TFA as a light yellow powder (800 mg, 95%): $[\alpha]_D$ = −1.6 (1.0% in DMSO); mp = 214−218 °C; IR (KBr): 3446, 2367, 1683, 1602, 1528, 1489, 1406, 1245, 1200, 1139, 1022, 833, 800; ^{1}H NMR (400 MHz) δ 1.45−1.70 (m, 4 H), 1.70−1.95 (m, 2H), 3.90 (m, 1H), 4.65 (m, 2H), 5.43 (s, 2H), 7.27 (m, 2H), 7.36 (m, 1H), 7.43 (t, J = 7.3 Hz, 2H), 7.52 (d, J = 7.4 Hz, 2H), 8.13 (d, J = 9.3 Hz, 1H), 7.90−8.70 (br.s., 3H), 8.90 (s, 1H), 11.00 (s, 1H, NH); ^{13}C NMR (100 MHz) δ 21.5, 25.9, 29.7, 43.6, 52.0, 70.1, 100.2, 112.0, 114.6, 115.9, 127.82, 128.2, 128.6, 133.7, 136.0, 141.1, 142.0, 156.8, 157.4, 162.3, 164.1, 171.0; HRMS calcd for $C_{24}H_{24}N_4O_5$ [MH^+] 449.1834, found 449.1824.

***(S)*-2-Amino-*N*$^\alpha$-(9-fluorenylmethyloxycarbonyl)-6-(8′-*O*-benzyl-5′-carbaisoalloxazin-10′-yl)hexanoic Acid ((*S*)-6).** *(S)*-2-Amino-6-(8′-*O*-benzyl-5′-carbaisoalloxazine-10′-yl)-hexanoic acid trifluoroacetic acid salt ((*S*)-**4**·TFA) (800 mg, 1.42 mmol) was dissolved in DMF (25 mL) and aqueous 10% $NaHCO_3$ solution. To the suspension was added Fmoc-OSu (600 mg, 1.78 mmol), dissolved in DMF (2 mL). The reaction suspension was shaken for 3 h at RT and aqueous 10% citric acid solution was carefully added until pH = 4 was reached. The mixture was diluted with $CHCl_3$ (200 mL), and the organic phase was separated. The aqueous phase was once again extracted with $CHCl_3$. The combined organic phases were washed with water, dried with $MgSO_4$, and evaporated in vacuo. Diethyl ether was added to the remaining oil in order to precipitate the product (*S*)-**6** as a light yellow powder. Recrystallization from methanol yielded the Fmoc-protected deazaflavin amino acid as a yellow powder (760 mg, 80%): $[\alpha]_D^{20}$ = −16.8 (1.0% in DMSO); mp = 135−138 °C; IR (KBr) 3433, 2367, 1700, 1603, 1561, 1529, 1444, 1400, 1239, 1161, 1083, 1028, 740; ^{1}H NMR (400 MHz) δ 1.42−1.55 (m, 2 H), 1.55−1.75 (m, 2 H), 1.75−1.85 (m, 2H), 4.00 (m, 1H), 4.65 (dd, J = 7.4 Hz, 14.5 Hz, 1H), 4.25 (m, 2H), 4.65 (br.s., 2H), 5.42 (s, 2H), 7.20−7.45 (m, 9H), 7.52 (d, J = 7.1 Hz, 2H), 7.64 (d, J = 7.9 Hz, 1H), 7.70 (t, J = 6.8 Hz, 2H), 7.88 (d, J = 7.5, 2H), 8.08 (d, J = 8.8 Hz, 1H), 8.87 (s, 1H), 10.95 (s, 1H), 12.5 (br.s., 1H); ^{13}C NMR (100 MHz) δ 23.0, 25.9, 30.5, 43.9, 46.53, 53.8, 65.5, 70.0, 100.0, 111.9, 114.7, 115.9, 120.0, 125.2, 126.9, 127.5, 127.7, 128.1, 128.5, 133.6, 135.9, 140.6, 141.0, 142.0, 143.7, 156.0, 156.6, 157.3, 162.2, 164.0, 173.7; MS (FAB$^+$) *m*/*z* (%) 671 (100) [M^+ + 1]. Anal. Calcd for $C_{39}H_{34}N_4O_7 \cdot 2H_2O$ (670.74 + 2(18.02)): C, 66.27; H, 5.42; N, 7.93. Found C, 66.44; H, 5.33; N, 7.82.

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