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Synthesis and peptide-binding properties of a luminescent pyrimidine zinc(II) complex

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Abstract—The synthesis and peptide-binding properties of a Zn(II)nitrilotriacetate complex substituted with pyrimidine hydrazine amides are reported. The metal complex provides millimolar binding affinity in aqueous buffer to peptides bearing N-terminal His. The pyrimidine heterocycles intermolecularly interact with the bound peptide and quench the emission of nearby Trp residues by energy transfer.

1. Introduction

Reversible interactions of ions or molecules by hydrogen bonds, electrostatic or van der Waals interactions are the foundation of molecular recognition processes.[1] However, the strength of hydrogen bonds and electrostatic interactions decreases rapidly as the polarity of the surrounding solvent increases.[2] This hampers the binding of substrates such as peptides, hormones, or carbohydrates under physiological conditions, which is of interest for medicinal applications and the design of biosensors. The use of reversibly coordinating metal complexes as binding sites is a suitable alternative, which may provide high affinity in competitive solvents.[3] Recent examples showed the ability of suitable metal complexes for selective binding to peptides[3c] and protein surface epitopes[4,5] under physiological conditions. Such synthetic receptors find use as bioanalytical probes[6] or markers[7] or can interfere with protein function, e.g., inhibiting enzyme activity[8] or protein–protein interactions.[9] We report here the use of a functionalized zinc(II) nitrilotriacetato (NTA) complex to label small peptides with pyrimidine hydrazino amides. Fluorescence resonance energy transfer (FRET)[10] from nearby Trp residues sensitizes an emission of the heteroaromatic pyrimidine ring.

2. Results and discussion

2.1. Synthesis

Various transition metal ion (e.g., Cu^{2+}, Ni^{2+}, and Zn^{2+}) complexes of NTA or IDA[11] bind to the imidazole side chains of surface exposed histidines of proteins.[12] This coordinative interaction is widely used for protein purification by immobilized metal affinity chromatography (IMAC)[13,14] and two-dimensional protein crystallization.[15] The dependence of the NTA binding constant on the divalent metal in $[M(NTA)]^-$ ($M=Mn^{2+}$, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}) has been intensively studied.[16] Although Ni^{2+} or Cu^{2+} NTA complexes show higher affinities to N-terminal His,[12] a Zn^{2+} complex[17,18] was chosen for peptide binding to obtain a diamagnetic compound, which allows NMR investigations. The synthesis of the peptide-binding Zn(II)–pyrimidine complex **6** is shown in Figure 1. As spacer between the complex and the heteroarene we choose a Gly unit to assist the possible formation of a hydrogen bond to a coordinated peptide. Compound **1**,[19] obtained from lysine methyl ester, is coupled to Boc-Gly-OH. After Boc deprotection, heterocycle **3**, which was reported recently,[3b] was introduced by standard peptide coupling procedures. Cleavage of the methyl ester under basic conditions generates the NTA ligand and complexation with Zn^{2+} leads to the desired functionalized complex **5**. To improve water solubility, the analogous complex **9**, extended by one pyrimidine hydrazine unit, was prepared (Fig. 2).

2.2. Structure

To derive structural information about the binding motif of **5** to the pentapeptide NH_2-His-Leu-Leu-Val-Phe-OMe

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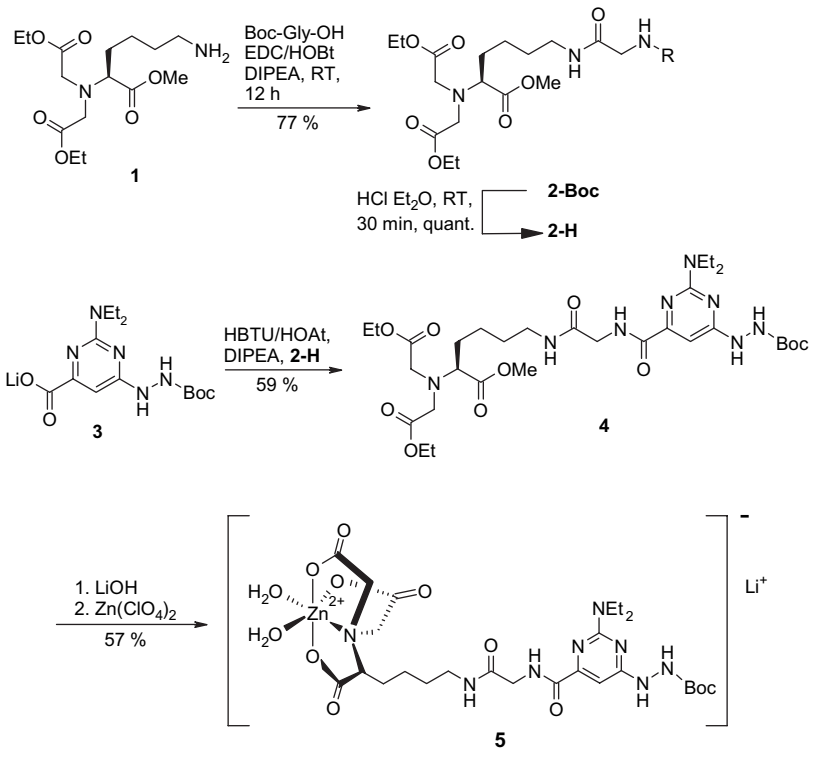

Figure 1. Synthesis of zinc(II)-NTA pyrimidine complex **5**.

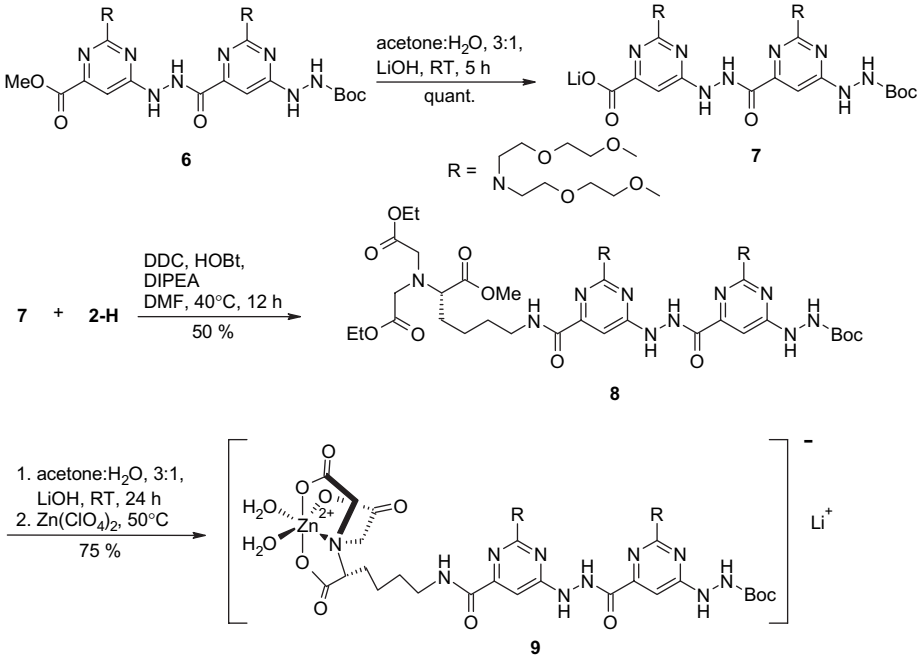

Figure 2. Synthesis of water-soluble zinc(II)-NTA pyrimidine complex **9**.

(Fig. 3) NMR experiments in DMSO-d_6 were performed.[20] Resonance signals of the NMR spectra of **5**—H-His-Leu-Leu-Val-Phe-OMe (c=3.3×10^{-2} M) were assigned (see Supplementary data, Fig. S-1 for details) and temperature-induced shift was used to identify hydrogen bonding of NH groups (see Supplementary data, Tables S-1, S-2 and Fig. S-2).[21] Shifts larger than −2 ppb/K typically indicate a strong interaction, while values smaller than −4 ppb/K show solvent exposed atoms.[22] The smallest ppb/K value (−2.57 ppb/K) in the aggregates spectrum was obtained for NH-**C**. This proton is most likely hydrogen bound to both the lone pair of the oxygen atom of the amide bond and the lone pair of the nitrogen atom in the pyrimidine ring. The temperature dependent shift of −2.95 ppb/K of NH-**G** indicates a hydrogen bond between peptide and complex. All other temperature dependent shifts of

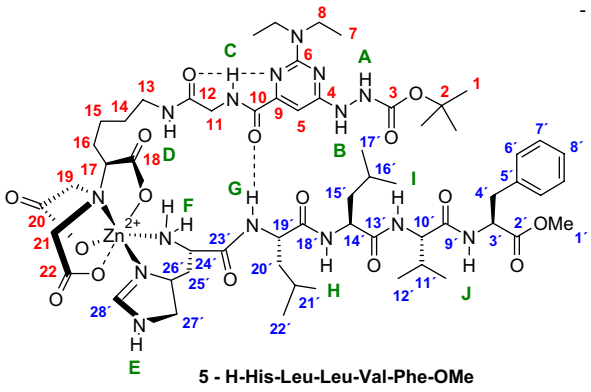

Figure 3. Structure and numbering of the proposed aggregate formed in an equimolar mixture of **5** and H-His-Leu-Leu-Val-Phe-OMe (c=3.3×10^{-2} M) in DMSO-d_6.

5—H-His-Leu-Leu-Val-Phe-OMe show values of 3 ppb/K or higher suggesting no strong hydrogen bonds.

2D-NOESY and ROESY[23] experiments (see Supplementary data, Figs. S-3 and S-4 for details) showed 10 contacts between functionalized complex **5** and the coordinated pentapeptide. The data support the depicted aggregate structure[24] (Fig. 3) with interactions of NH-**C** and protons at C-11 to NH-**G**, NH-**H** and protons at C-19′ to C22′, and contacts of imidazole and parts of the NTA ligand. A similar aggregate analysis was attempted using complex **9** and H-His-Asp-Trp-Ser-Gly-OH in buffered water. Resonance signals of the individual compounds were assigned and their chemically induced shift in the mixture indicates interactions of complex and peptide (see Supplementary data, Figs. S-5 and S-6). However, substantial signal broadening in the spectrum of the mixture did not allow a more detailed analysis.

2.3. Peptide binding

The Trp emission of peptides is quenched upon their coordination to complex **9**. A perfect overlap of the pyrimidine absorption spectrum with the Trp emission allows intramolecular energy transfer (Fig. 4). The FRET emission of the pyrimidine chromophore is visible in aprotic solvents, such as acetonitrile, but weak in aqueous media. Therefore, Trp emission quenching (Fig. 5) was used to monitor the binding of complex **9** to pentapeptides H-His-Asp-Trp-Ser-Gly-OH and H-His-Thr-Trp-Asp-Asp-OH.

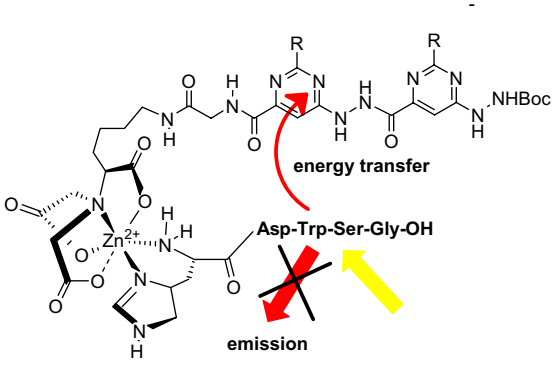

Figure 4. Intramolecular energy transfer within the peptide–metal complex aggregate leading to Trp emission quenching.

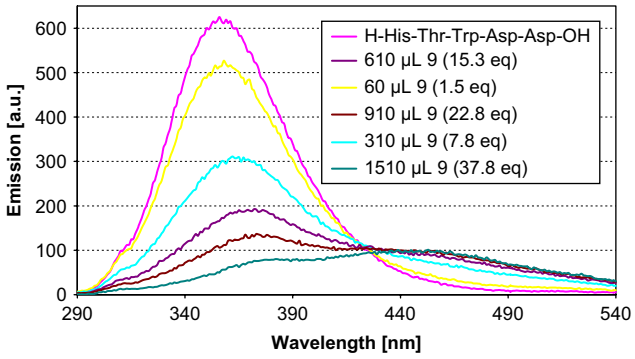

Figure 5. Fluorescence titration of H-His-Thr-Trp-Asp-Asp-OH (1.0×10^{-5} mol/L) with compound **9** in TRIS buffered aqueous solution at pH 7.2; λ_{ex}=280 nm.

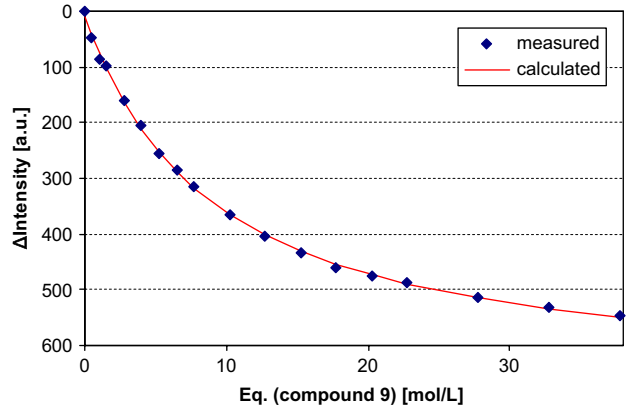

Figure 6. Fluorescence titration of H-His-Thr-Trp-Asp-Asp-OH (1.0×10^{-5} mol/L) with compound **9** in Tris buffered aqueous solution at pH 7.2; λ_{ex}=280 nm.

A binding stoichiometry of 1:1 for complex **9** and the peptides was confirmed by Job's plot analysis (see Supplementary data, Figs. S-7 and S-9 for data). Emission titration data were used to derive binding affinities, which are, as expected for the complexation of N-terminal histidine to a Zn(II)-NTA, in the millimolar range. The binding affinity of **9** to H-His-Asp-Trp-Ser-Gly-OH (log K=4.6±0.3 L/mol; Fig. S-8) is slightly higher than the value for H-His-Thr-Trp-Asp-Asp-OH (log K=4.0±0.3 L/mol; Fig. 6).[25] Ligand **8** or Trp-containing peptides missing N-terminal histidine show no affinity under the experimental conditions, confirming the importance of the Zn(II)-NTA to His complexation for the binding. The addition of a non-substituted Zn(II)-NTA complex or pyrimidine amino acids does not affect the Trp emission.

3. Conclusion

The combination of an imidazole-coordinating metal complex, which binds to N-terminal His, with luminescent pyrimidine hydrazine acids (PHA) leads to a luminescent non-covalent peptide label. The proposed binding process occurs in two steps. Initially, the Zn(II)-NTA complex strongly coordinates to the imidiazole of an N-terminal His, followed by weaker intramolecular interaction of the PHA moiety to the backbone of the peptide. Within the aggregate, quenching of Trp peptide emission by energy transfer to the PHA moieties signals the binding process. Zn(II)-NTA–PHA

complexes like **9** may find use as molecular probes to explore peptidic structures in physiological solution.

4. Experimental

4.1. 2-(Bis-ethoxycarbonylmethyl-amino)-6-(2-*tert*-butoxycarbonylamino-acetylamino)-hexanoic acid methyl ester (2-Boc)

To a solution of 131 mg (0.75 mmol) Boc-Gly-OH, 122 mg (0.9 mmol) HOBt, 158 μL (140 mg, 0.9 mmol) NEt_3, and 447 μL (339 mg, 2.63 mmol) Huenig's base in 2 mL of DMF was added a solution of 302 mg (0.75 mmol) amine **1** in 1 mL of DMF at 0 °C. The reaction mixture was allowed to warm to rt and stirred for 18 h. The solution was diluted with H_2O (4 mL) and extracted with CH_2Cl_2 (2×10 mL). The organic phase was dried over Na_2SO_4, evaporated, and then concentrated under reduced pressure. The residue was purified by column chromatography (EtOAc, R_f=0.36) to afford 280 mg of **2-Boc** as a colorless oil in 76% yield. IR (KBr disk) cm^{-1}: 3362, 2981, 2938, 2251, 1740, 1670, 1166. ^1H NMR ($CDCl_3$, 400 MHz): δ=1.28 (t, ^3J=7.1 Hz, 6H), 1.47 (s, 9H), 1.48–1.67 (m, 4H), 1.70–1.74 (m, 2H), 3.28–3.31 (m, 2H), 3.45 (t, ^3J=7.6 Hz, 1H), 3.61 (s, 2H), 3.62 (s, 2H), 3.71 (s, 3H), 3.81–3.85 (m, 2H), 4.17 (q, ^3J=7.1 Hz, 4H), 5.42 (br s, 1H), 6.51 (br s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ=14.2 (+), 22.5 (−), 28.2 (−), 28.3 (+), 29.3 (−), 39.1 (−), 44.2 (−), 51.4 (+), 52.7 (−), 60.7 (−), 64.1 (+), 79.9 (C_{quat}), 156.0 (C_{quat}), 169.5 (C_{quat}), 171.5 (C_{quat}), 173.3 (C_{quat}). MS (ESI, DCM/MeOH+10 mmol/L NH_4Ac): *m/z* (%)=490.3 [M+H^+] (100). HRMS calcd for $C_{22}H_{39}N_3O_9$: 489.2686; found: 489.2680±0.0004.

4.2. 6-(2-Amino-acetylamino)-2-(bis-ethoxycarbonyl-methyl-amino)-hexanoic acid methyl ester dihydrochloride (2-H)

Compound **2-Boc** (259 mg, 0.53 mmol) was dissolved in 3 mL of ether saturated with HCl. The solution was stirred for 15 h. The precipitate was filtered off, washed with cold ether, and dried in vacuum to afford the deprotected amine **2-H** as a colorless, hygroscopic salt in quantitative yield (242 mg). The salt was used for subsequent reactions without further purification. Mp: >200 °C (decomp.). IR (KBr disk) cm^{-1}: 3423, 2955, 1747, 1656, 1558, 1378, 1224, 1019, 914, 706. ^1H NMR (DMSO-d_6, 300 MHz): δ=1.17 (t, ^3J=7.0 Hz), 1.30–1.48 (m, 4H), 1.51–1.63 (m, 2H), 3.01–3.14 (m, 2H), 3.32–3.42 (m, 1H), 3.34–3.54 (m, 4H), 3.55–3.61 (m, 5H), 4.04 (q, ^3J=7.0 Hz, 4H), 8.16–8.34 (m, 3H), 8.48–8.52 (m, 1H), 9.94 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 150 MHz): δ=15.1 (+), 22.6 (−), 28.4 (−), 29.2 (−), 38.4 (−), 39.9 (−), 51.0 (+), 52.1 (−), 59.0 (−), 63.7 (+), 165.5 (C_{quat}), 170.7 (C_{quat}), 172.3 (C_{quat}). MS (ESI, DCM/MeOH+10 mmol/L NH_4Ac): *m/z* (%)=390.2 [M+H^+] (100).

4.3. 2-(Bis-ethoxycarbonylmethyl-amino)-6-(2-{[6-(*N′*-*tert*-butoxycarbonyl-hydrazino)-2-diethylamino-pyrimidine-4-carbonyl]-amino}-acetylamino)-hexanoic acid methyl ester (4)

A solution of 120 mg (0.26 mmol) of the deprotected amine **2-H**, 86 mg (0.26 mmol) of **3**, 70 mg (0.52 mmol) HOBt, 197 mg (0.52 mmol) HBTU and 224 μL (168 mg, 1.3 mmol) of Huenig's base in 4 mL of DMF was stirred for 24 h at rt. The solution was cooled to 0 °C, diluted with cold H_2O (5 mL), and extracted with CH_2Cl_2 (3×10 mL). The organic phase was dried over $MgSO_4$, evaporated, and then concentrated under reduced pressure. The residue was purified by column chromatography (EtOAc, R_f=0.3) to afford **4** as a white solid in 59% (106 mg) yield. Mp: 144 °C; IR (KBr disk) cm^{-1}: 3325, 2989, 1735, 1649, 1532, 1380, 1187, 1093. ^1H NMR ($CDCl_3$, 600 MHz): δ=1.17 (t, ^3J=6.9 Hz, 6H), 1.22–1.28 (m, 6H), 1.35–1.45 (m, 2H), 1.47 (s, 9H), 1.55–1.60 (m, 2H), 1.66–1.72 (m, 2H), 3.23–3.33 (m, 2H), 3.40 (t, ^3J=7.6 Hz, 1H), 3.58–3.62 (m, 8H), 3.67 (s, 3H), 4.07–4.16 (m, 6H), 6.45–6.75 (m, 2H), 6.65 (br s, 1H), 6.69 (br s, 1H), 8.51 (t, ^3J=5.0 Hz, 1H). ^{13}C NMR ($CDCl_3$, 150 MHz): δ=13.2 (+), 14.2 (+), 22.9 (−), 28.2 (+), 28.4 (−), 29.6 (−), 39.1 (−), 41.9 (−), 43.2 (−), 51.4 (+), 52.7 (−), 60.6 (−), 64.6 (+), 81.7 (C_{quat}), 115.4 (+), 155.7 (C_{quat}), 155.8 (C_{quat}), 156.9 (C_{quat}), 160.1 (C_{quat}), 164.8 (C_{quat}), 168.7 (C_{quat}), 171.5 (C_{quat}), 173.2 (C_{quat}). MS (ESI, MeOH+10 mmol/L NH_4Ac): *m/z* (%)=719.4 [M+Na^+] (22), 697.4 [MH^+] (100).

4.4. Zn-NTA-complex 5

Seventy-three milligrams (0.1 mmol) of ester **4** and 3 mg (0.30 mmol) of LiOH were dissolved in a 4:1 acetone/water mixture and stirred for 1 day at 40 °C. The solvents were removed under reduced pressure to afford the hygroscopic 2-(bis-carboxymethyl-amino)-6-(2-{[6-(*N′*-*tert*-butoxycarbonyl-hydrazino)-2-diethylamino-pyrimidine-4-carbonyl]-amino}-acetylamino)-hexanoic acid Li salt (61 mg, 95%) in almost quantitative yield. The salt was used for complexation without further purification. Mp: >250 °C (decomp.). ^1H NMR (D_2O, 300 MHz): δ=0.52–0.73 (m, 6H), 0.86–1.69 (m, 15H), 2.78–3.22 (m, 5H), 3.34–3.63 (m, 4H), 3.84–4.06 (m, 4H), 6.34 (s, 1H), 7.12–7.24 (m, 1H), 7.34–7.45 (m, 1H). MS (ESI, H_2O/MeCN/MeOH+10 mmol/L NH_4Ac): *m/z* (%)=391.3 [M+H^+] (100), 408.2 [M+NH_4^+] (22), 798.7 [2M+NH_4^+] (37), 803.6 [2M+Na^+] (23).

Sixty-four milligrams (0.10 mmol) of the lithium salt were suspended in 10 mL of H_2O and 13.6 mg (0.10 mmol) of $ZnCl_2$ was added. The reaction mixture was stirred for 45 min at 40 °C, filtered and the solvent was removed under reduced pressure. The solid was dissolved in EtOH and treated with hexane precipitating **5** in 65% (47 mg) yield. IR (KBr disk) cm^{-1}: 3412, 2987, 2944, 2880, 1931, 1605, 1537, 1418, 1264, 965, 820. ^1H NMR (MeOH-d_4, 600 MHz): δ=1.15 (t, ^3J=7.0 Hz, 6H), 1.29–1.68 (m, 15H), 3.03–3.21 (m, 3H), 3.31–3.34 (m, 4H), 3.63 (q, ^3J=7.0 Hz, 4H), 4.05–4.10 (m, 2H), 6.52 (s, 1H). ^{13}C NMR (MeOH-d_4, 150 MHz): δ=13.8 (+), 26.8 (−), 28.3 (−), 28.7 (+), 30.3 (−), 42.8 (−), 43.6 (−), 55.7 (−), 60.3 (−), 69.1 (+), 81.7 (C_{quat}), 91.8 (+), 157.7 (C_{quat}), 158.7 (C_{quat}), 161.9 (C_{quat}), 167.3 (C_{quat}), 171.1 (C_{quat}), 178.1 (C_{quat}), 178.3 (C_{quat}), 179.5 (C_{quat}), 180.5 (C_{quat}). MS (ESI, H_2O/MeOH+10 mmol/L NH_4Ac): *m/z* (%)=687.4 [M−H^+]$^-$ (100).

4.5. Dipyrimidine 7

To a solution of **6** (200 mg, 0.24 mmol) in 4 mL of water/acetone (v/v, 3:1) was added $LiOH\cdot H_2O$ (11.5 mg,

0.27 mmol). The reaction mixture was stirred for 6 h at rt. Acetone was removed in vacuum and the remaining solvent was lyophilized. Compound **7** (200 mg) was obtained quantitatively. IR ($CHCl_3$) cm^{-1}: 3423, 1668, 1618, 1529, 1374, 1084, 668. ^1H NMR ($CDCl_3$, 300 MHz): δ=1.34 (s, 9H), 3.19 (m, 12H), 3.40–3.44 (m, 8H), 3.52–3.58 (m, 8H), 3.62–3.67 (m, 8H), 3.69–3.72 (m, 8H), 6.36 (s, 1H), 6.46 (s, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ=27.5, 46.7, 46.9, 58.0, 68.6, 69.3, 69.4, 70.9, 71.0, 82.4, 158.2, 161.5. UV (MeCN) λ_{max} (log ε): 336 (6.76).

4.6. Compound 8

A mixture of **7** (200 mg, 0.24 mmol), **2-H** (94 mg, 0.28 mmol), HOBt (96 mg, 0.71 mmol), EDC (110 mg, 0.71 mmol), and DIPEA (153 mg, 1.18 mmol) in 8 mL of DMF was stirred for 12 h at 40 °C. The solution was allowed to cool to rt, solvents were removed in vacuum and the crude product was purified by column chromatography (CH_2Cl_2/MeOH 1:40, R_f=0.30) to afford compound **8** as a yellow oil (135 mg, 50%). IR ($CHCl_3$) cm^{-1}: 3295, 1639, 1583, 1506, 1406, 1091. ^1H NMR ($CDCl_3$, 300 MHz): δ=1.23 (t, J=7.2 Hz, 6H), 1.32–1.40 (m, 2H), 1.44 (s, 9H), 1.59–1.71 (m, 4H), 3.29–3.39 (m, 13H), 3.48–3.51 (m, 10H), 3.57–3.60 (m, 10H), 3.65–3.68 (m, 8H), 3.78–3.80 (m, 10H), 4.07–4.14 (m, 4H), 6.98 (s, 1H), 7.29 (s, 1H), 7.46 (s, 1H, NH), 8.73 (s, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ=14.2, 23.3, 28.2, 28.7, 30.3, 40.1, 51.4, 52.6, 58.8, 60.6, 64.7, 69.4, 70.4, 71.7, 71.8, 81.6, 155.5, 171.5, 173.1. UV (MeCN): λ_{max} (log ε): 332 (6.05). MS (ESI, MeOH+10 mmol/L NH_4Ac): m/z (%)=1143.8 [MH^+] (26), 572.5 [M+2H^+] (100). HRMS calcd: 1143.6261 [M^+]; found: 1143.6289.

4.7. Complex 9

To a solution of **8** (130 mg, 0.12 mmol) in 3 mL of water/acetone (v/v, 3:1) was added $LiOH \cdot H_2O$ (15.2 mg, 0.36 mmol). The reaction mixture was stirred for 24 h at rt. Acetone was removed in vacuum and the remaining solution was lyophilized. The residue and zinc carbonate (29 mg, 0.05 mmol) were dissolved in H_2O (20 mL). After stirring for 1 h the suspension was heated to 55 °C for 24 h. Insoluble particles were filtered off and the filtrate was lyophilized. The raw product was dissolved in ethanol and ether was added. The precipitated material was separated from solution by centrifugation to give **9** (97 mg, 75%). IR ($CHCl_3$) cm^{-1}: 3282, 2930, 1722, 1585, 1511, 1431, 1369, 1249, 1161, 1096, 847, 782. ^1H NMR ($CDCl_3$, 300 MHz): δ=1.35 (s, 9H), 1.43–1.52 (m, 4H), 1.72–1.80 (m, 2H), 3.07–3.19 (m, 16H), 3.26–3.44 (m, 16H), 3.55–3.56 (m, 8H), 3.64–3.73 (m, 10H), 6.45 (s, 1H), 6.54 (s, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ=28.1, 47.3, 56.8, 68.3, 69.3, 69.4, 71.0, 160.8, 176.2. UV (MeCN) λ_{max} (log ε): 338 (6.06). MS (ESI, MeOH+10 mmol/L NH_4Ac): m/z (%)=1133.7 [MH^+] (80), 566.4 [M+2H^+] (100). HRMS calcd: 1135.4614 [M^-+2H]$^+$; found: 1135.4586.

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Supplementary data

Spectroscopic investigations of the aggregate of **5**—H-His-Leu-Leu-Val-Phe-OMe and **9**—H-His-Asp-Trp-Ser-Gly-OH. Analysis of the binding of compound **9** with H-His-Asp-Trp-Ser-Gly-OH and H-His-Thr-Trp-Asp-Asp-OH in aqueous buffer. Copies of proton NMR spectra of compounds **7–9**. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2006.10.019.

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