Scientific paper

Synthesis of the Peptide-Based Phenanthridine-Nucleobase Conjugates and Study of Their Interactions With ds-DNA

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Dedicated to Prof. Dr. Gorazd Vesnaver on the occasion of his 70th birthday

Abstract

Series of novel peptide-bridged phenanthridine-nucleobase conjugates were prepared by solid phase peptide synthesis, which allowed easy and fast tuning of structure properties. Compounds were fully characterized in aqueous medium, pointing out to intramolecularly stacked structures. The stacked phenanthridine-thymine-phenanthridine system revealed characteristic excimeric fluorescence band and very specific CD spectrum. Studied compounds interact with double stranded DNA by intercalation, whereby binding is to minor extent influenced by attached thymine and amino-acid sequence of the peptide backbone.

Keywords: Phenanthridine-nucleobase conjugate, DNA, CD, fluorescence

1. Introduction

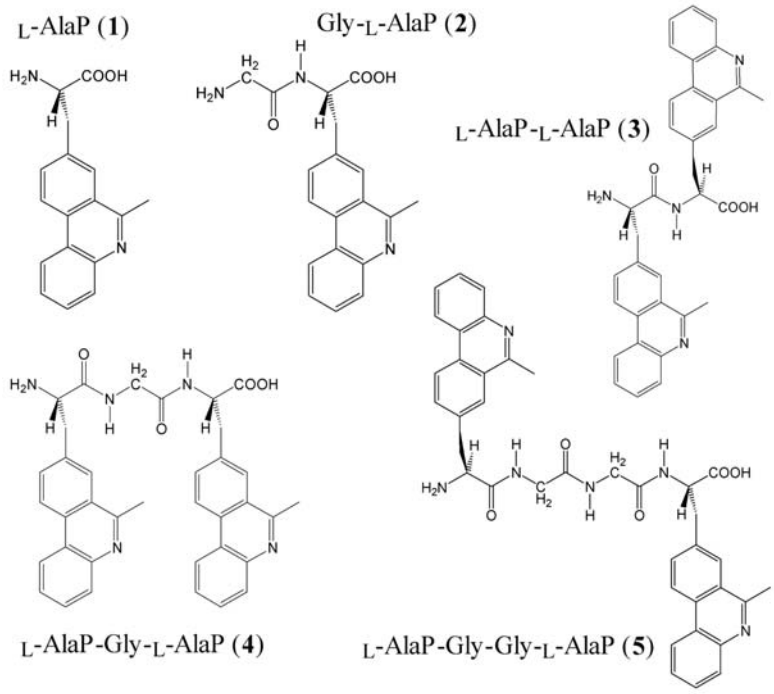
The recognition of specific nucleic acid sequences by novel small molecules has been the subject of a large number of studies over many years.[1] Moreover, small molecules emitting specific or at least highly selective signals upon binding to certain DNA/RNA sequences are important for many technologies used in the molecular biology and medicine.[2] Small organic molecules can bind to DNA by means of a non-specific (mainly electrostatic) binding along the DNA exterior, a specific groove binding and intercalation which is characterized by insertion of planar aromatic molecules between base pairs and is generally independent of base pair sequence.[3] Phenanthridine moiety is often applied for an "aryl" component due to characteristic structural features like high polarizability, high electron affinity and highly polar amino groups.[4] Furthermore, by taking advantage of weakly acidic pK of phenanthridine heterocyclic nitrogen, it was possible to efficiently and reversibly control some interactions with DNA/RNA by external stimuli (pH), as it was previously shown for aliphatic amine –phenanthridine conjugates.[5]

One of the most extensively studied groups of DNA/RNA targeting compounds are intercalator–nucleobase conjugates, whereby the intercalator should "anchor" the compound to the ds-DNA or ds-RNA by the high affinity of aromatic stacking interactions, while nucleobase should yield the recognition of the nearby abasic site by insertion and formation of hydrogen bonds with the complementary nucleobase.[6,7] Lhomme et al showed the capacity of aryl –nucleobase conjugates to recognize certain nucleobases in water,[8] while Kimura et al demonstrated that zinc(II) complexes of the macrocyclictetraamine 1,4,7,10-tetraazacyclododecane (cyclen) have a unique propensity to bind with deprotonated imides like thymine and, uracil, by forming non-covalent stable complexes in biologically relevant conditions.[9,10]

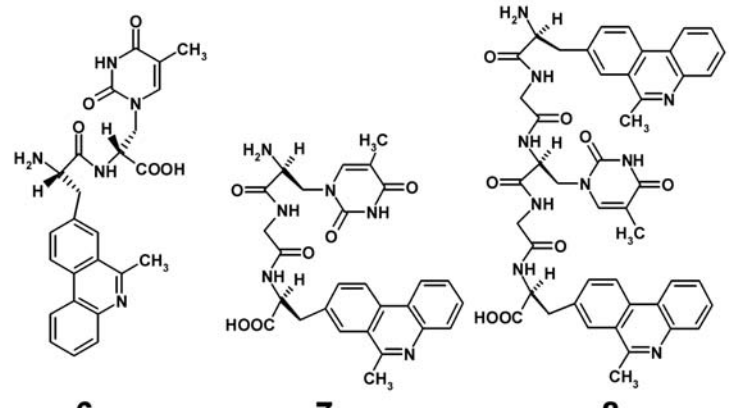
A number of phenanthridinium–nucleobase conjugates characterized byinert aliphatic linkers interacted selectively with complementary polynucleotide sequences, whereby the polynucleotide hydrophobic environment most likely allowed formation of specific hydrogen bonds between nucleobase attached to intercalator and nucleobases of polynucleotide.[11,12] Very recently we showed that covalently attaching the nucleobase to the bis-intercalator

skeleton by linkers, which would allow insertion of nucleobase between two intercalative subunits, could result in selective interactions of bis-phenanthridine-adenine conjugate with complementary UMP.[13] These results pointed out that high stability and selectivity toward specific single stranded DNA/RNA sequences could be achieved by similar principle because hydrophobic cleft between two phenanthridine subunits formed excellent recognition spot for complementary ss- sequence, whereby basepair (formed of nucleobase covalently attached to bis-phenanthridine and nucleobase of ss-polynucleotide) would be additionally stabilized by aromatic stacking interactions.

In the design of novel bis-phenanthridine-nucleobase conjugates (Scheme 2) we decided to use convergent synthetic route to an easily variable spacer backbone, by applying solid phase peptide synthesis procedures.[14,15] The most prominent advantages of the convergent approach in comparison to previously used consequtive[12,13] are: a) an easy preparation of novel amino acid-phenanthridine monomer; b) oligomerization of monomers can be easily controlled by means of order, chirality of linker, distance between monomers; c) simple and fast preparation of new generations of compounds by modification of the last (oligomerization) step.



Scheme 1. Previously studied peptide-phenanthridine conjugates.[15]



Scheme 2. Novel phenanthridine– thymine conjugates **6**-**8**.

2. Experimental

2. 1. Materials and Methods

The HPLC analyses were performed on Phenomenex Onyx Monolythic C-18, 4.6 mm × 100 mm analytical column. For purifications Waters Xterra Prep RP_{18}, 5 μm, 19 × 100 mm preparative column was used. Pure products were characterized by ESI MS and detailed structural analysis as well as determination of purity (≈ 98%) was done by ^{1}H NMR and ^{13}C NMR, as well as by Hetcor and HMBC spectra (Bruker AV600 at 600 MHz) at 25°C. Chemical shifts (δ) were given in parts per million (ppm) relative to tetramethylsilane as an internal standard and coupling constants (J) in Hz. The splitting patterns in the ^{1}H NMR spectra are denoted as follows: s (singlet), d (doublet), t (triplet), pst (pseudotriplet), m (multiplet).

2. 2. Synthesis

General procedure for oligomerization

Final compounds **6-8** were prepared from N^{α}-(tert-butoxycarbonyl)-3-(6-methylphenanthridine-8-yl)-L-alanine,[16][N^{α}-(tert-butoxycarbonyl)-β-thymine-1-yl]D-or L-alanine[17]and corresponding N-Boc-protected glycine, by application of solid-phase peptide synthesis on PL-MBHA resin (1% divinylbenzene polystyrene, loading 1.2 mmol/g 4-methylbenzhydrilamine hydrochloride) in disposable PE syringe-style reaction vessels fitted with a PE plug, a PTFE filter frit and a luer cap.[15] Agitation of the reaction mixture was controlled by a mechanical shaker device. Draining the solvents from the resin was done by suction. The quantities of solvents are given on the scale of 50 mg (0.06mmol MBHA, 1 equiv.) of PL-MBHA resin used and were scaled up or scaled down as needed. Deprotectionmixture was 25% TFA and 2% anisole in DCM, cleavage mixture was 10% TFMSA acid and 10% *m*-cresol in TFA. The synthesis was performed in cycles (one for each amino acid), the cycle consisting of the following steps:

a) Preparing the resin (first cycle only). The resin was placed in a reaction vessel, dry DMF (1.0 mL) was added, reaction mixture shaken for 5 min and allowed to swell for 30 min. DMF was drained.

b) Activating the Boc-protected amino acid. Boc-protected amino acid (0.18 mmol, 3.0 equiv.), HBTU (63 mg, 0.17 mmol, 2.8 equiv.) and HOBt (24 mg, 0.18 mmol, 3.0 equiv.) were dissolved in dry DMF (1 mL). DIEA (57 μL, 0.41 mmol, 7.0 equiv.) was added and the reaction mixture was shaken gently for 5 min. Alternatively, HATU (63 mg, 0.17 mmol, 2.8 equiv.) was used instead of HBTU and HOBt.

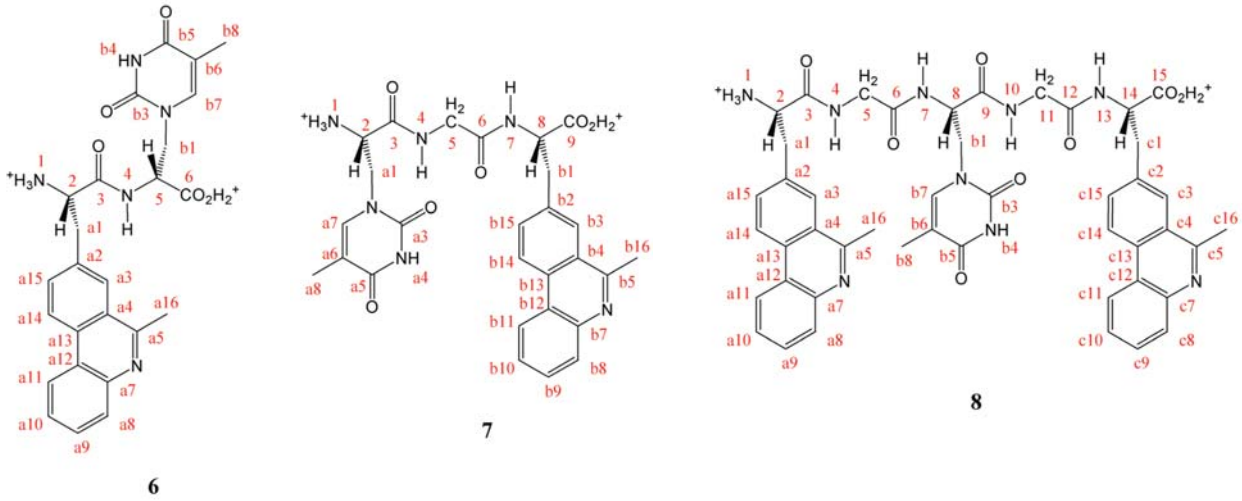
c) Coupling. The above activated amino acid solution was added to the resin and the reaction vessel shaken for 1.5 h up to 4 days, depending on e). The solvent was drained.

d) Washing 1. DMF (4–5 mL) was added to the resin, the reaction vessel shaken for 2–3 min and the solvent drained, repeating this 3 times. Dichloromethane (4–5 mL) was added to the resin, the reaction vessel shaken for 2–3 min and the solvent drained, repeating this 3 times.

e) Coupling completion test. About 1 mg of resin was sampled and treated with 100 μL of the cleavage mixture for 1 h, diluted with MeCN/water (1:1) and analysed by C-18 reverse phase HPLC-MS. If the coupling was judged incomplete, the main part of the reaction was submitted to cycle b) followed by prolonged c).

f) Deprotection. Deprotection mixture (1–2 mL) was added to the resin, shaken for 2–3 min, drained, another portion of deprotection mixture (1 mL) was added, shaken for 30 min and drained.

g) Washing 2. Dichloromethane (4–5 mL) was added to the resin, the reaction vessel shaken for 2–3 min and the solvent drained, repeating this 3 times. DMF (4–5 mL) was added to the resin, the reaction vessel shaken for 2–3 min and the solvent drained, repeating this 3



Scheme 3. Structures of final compounds assigned for NMR analysis.

times. If another amino acid monomer had to be added, the reaction was submitted to cycle b).

h) Washing 3 (last cycle only). Dichloromethane (4–5 mL) was added to the resin, the reaction vessel shaken for 2–3 min and the solvent drained, repeating this 3 times. Diethyl-ether (3–4 mL) was added to the resin, the reaction vessel shaken for 2–3 min and the solvent drained, repeating this 2 times.

i) Cleavage from the resin (last cycle only). Cleavage mixture (1.5 mL) was added to the resin and the reaction vessel shaken for 1 h. The black solution was drained from the resin into the sample collection vial.

Workup procedure

To each sample cold diethyl-ether was added, the precipitate spun down in a centrifuge and the clear solvent discarded, repeating this 4 times. The precipitate was dried at high vacuum to remove traces of solvents. Samples which did not precipitate were concentrated at reduced pressure at 40°C. All crude products were purified on a C-18 reverse phase preparative HPLC-MS system using custom gradients of MeCN in water (containing 0.1% formic acid). The collected fractions were lyophilized to obtain final compounds as white foam.

*L-AlaP-D-AlaT (**6**)*

^{1}H NMR (DMSO-d$_6$, *c*(**6**) = 2.7 mmol dm^{-3}) 1.72 (s, 3H, H_3C-b8), 2.91 (m, 1H, HH'C-a1), 3.02 (s, 3H, H_3C-a16), 3.45 (m, HH'C-a1), 3.83 (dd, 1H, $^3J_{HH}$ = 9.8 Hz, $^2J_{HH}$ = 13.8 Hz, HH'C-b1), 4.04 (dd, 1H, $^3J_{HH}$ = 3.8 Hz, $^2J_{HH}$ = 13.8 Hz, HH'C-b1), 4.26 (bs, 1H, HC-2), 4.85 (ddd m, 1H, HC-5), 7.47 (s, 1H, HC-b7), 7.52 (s, 1H, HOC-6), 7.72 (dd, 1H, HC-a10), 7.78 (dd as broad s, 1H, HC-a9), 7.80 (s, 1H, H'O'C-6), 7.89 (d, 1H, $^3J_{HH}$ = 7.1 Hz, HC-a15), 8.02 (s, 3H, H_3N-1), 8.04 (d, 1H, $^3J_{HH}$ = 9.1 Hz, HC-a8), 8.29 (s, 1H, HC-a3), 8.79 (d, 1H, $^3J_{HH}$ = 6.8 Hz, HC-a11), 8.88 (d, 1H, $^3J_{HH}$ = 7.7 Hz, HC-a14), 8.97 (d, 1H, $^3J_{HH}$ = 8.9 Hz, HN-4), 11.34 (s, 1H, HN-b4).

^{13}C NMR (DMSO-d$_6$, *c*(**6**) = 2.7 mmol dm^{-3}) 12.0 (C-b8), 22.5 (C-a16), 36.9 (C-a1), 49.1 (C-b1), 51.2 (C-5), 53.7 (C-2), 108.3 (C-b6), 122.7 (C-a11), 123.2 (C-a12 and/or C-a4), 123.3 (C-a14), 127.8 (C-a3), 129.0(C-a9), 141.6 (C-b7), 151.0 (C-b3), 164.2 (C-b5), 168.4 (C-3), 170.0 (C-6).

MS (ESI) 127.1 (32%), 168.2 (11%), 235.2 (22%), 475.1 (100%, [M + H]$^+$).

*L-AlaT-Gly-L-AlaP (**7**)*

^{1}H NMR (DMSO-d$_6$, *c*(**7**) = 3.3 mmol dm^{-3}) 1.62 (s, 3H, H_3C-a8), 2.97 (s, 3H, H_3C-b16), 3.05 (dd, 1H, $^3J_{HH}$ = 9.8 Hz, $^2J_{HH}$ = 13.5 Hz, HH'C-b1), 3.32 (dd, HH'C-b1), 3.81 (4 dd, 4H, H_2C-a1, H_2C-5), 3.95 (bs, 1H, HC-2), 4.64 (ddd, 1H, HC-8), 7.20 (s, 1H, HOC-9), 7.28 (s, 1H, HC-a7), 7.5 – 8.5 (bs, H_3N-1), 7.59 (s, 1H, H'O'C-9), 7.63 (dd, 1H, $^3J_{HH}$ = 7.5 Hz, HC-b10), 7.69 (dd, 1H, $^3J_{HH}$ = 7.5 Hz, HC-b9), 7.83 (d, 1H, $^3J_{HH}$ = 8.4 Hz, HC-b15), 7.96 (d, 1H, $^3J_{HH}$ = 8.0 Hz, HC-b8), 8.34 (s, 1H, HC-a3), 8.40 (d, 1H, $^3J_{HH}$ = 8.5 Hz, HN-7), 8.66 (s, 1H, HN-4), 8.70 (d, 1H, $^3J_{HH}$ = 7.7 Hz, HC-b11), 8.73 (d, 1H, $^3J_{HH}$ = 8.6 Hz, HC-b14).

^{13}C NMR (DMSO-d$_6$, *c*(**7**) = 3.3 mmol dm^{-3}) 11.8 (C-a8), 23.0 (C-b16), 37.9 (C-b1), 41.8 (C-5), 47.9 (C-a1), 51.8 (C-2), 53.8 (C-5), 108.8 (C-a6), 122.4 (C-b11), 122.5 (C-b14), 123.2 (C-b12), 125.2 (C-b4), 126.4 (C-b10), 126.9 (C-b3), 128.4 (C-b9), 128.8 (C-b8), 130.4 (C-b13), 132.2 (C-b15), 137.6 (C-b2), 141.0 (C-a7), 143.0 (C-b7), 151.4 (C-a3), 158.5 (C-b5), 164.3(C-a5), 167.8 (C-6 and/or C-3), 172.6 (C-9).

MS (ESI) 125.1 (14%), 127.1 (33%), 168.2 (100%), 235.5 (13%), 258.3 (72%), 280.2 (10%), 337.2 (60%), 532.2 (21%, [M + H]$^+$).

*L-AlaP-Gly-L-AlaT-Gly-L-AlaP (**8**)*

^{1}H NMR (DMSO-d$_6$, *c*(**8**) = 5.5 mmol dm^{-3}) 1.66 (s, 3H, H_3C-b8), 3.00 (s, 3H, H_3C-a16 or H_3C-c16), 3.04 (bs, 3H, H_3C-c16 or H_3C-a16), 3.09 (dd, $^3J_{HH}$ = 9.8 Hz, $^2J_{HH}$ = 13.9 Hz, HH'C-c1), 3.21 (dd, $^3J_{HH}$ = 8.8 Hz, $^2J_{HH}$ = 13.9 Hz, HH'C-a1), 3.34 (m, HH'C-c1), 3.38 (m, HH'C-a1), 3.63 (m, 1H, HH'C-11 or HH'C-5), 3.63 (m, 1H, HH'C-b1), 3.73 (m, 1H, HH'C-5 or HH'C-11), 3.77 (m, 1H, HH'C-11 or HH'C-5), 3.94 (m, 1H, HH'C-5 or HH'C-11), 3.96 (m, HH'C-b1), 4.22 (bs, 1H, HC-2), 4.62 (ddd, 1H, HC-14), 4.63 (ddd, 1H, HC-8), 7.21 (s, 1H, HOC-15), 7.28 (s, 1H, HC-b7), 7.56 (s, 1H, H'O'C-15), 7.70 (m, 2H, HC-a10, HC-c10), 7.77 (m, 2H, HC-a9, HC-c9), 7.86 (d, 1H, $^3J_{HH}$ = 7.4 Hz, HC-a15 or HC-c15), 7.90 (d, 1H, $^3J_{HH}$ = 7.4 Hz, HC-c15 or HC-a15), 8.02 (d, 2H, $^3J_{HH}$ = 7.8 Hz, HC-a8, HC-c8), 8.11 8.13 8.15 (m, 3H, H_3N-1), 8.25 (m, 3H, HC-a3, HC-c3, HN-13), 8.31 (d, 1H, $^3J_{HH}$ = 8.5 Hz, HN-7), 8.37 (t, 1H, $^3J_{HH}$ = 5.6 Hz, HN-10 or HN-4), 8.79 (m, 5H, HN-4 or HN-10, HC-a11, HC-c11, HC-a14, HC-c14), 11.20 (s, 1H, HN-b4).

^{13}C NMR (DMSO-d$_6$, *c*(**8**) = 5.5 mmol dm^{-3}) 11.8 (C-b8), 22.5 (C-a16 or C-c16), 37.0 (C-c1), 37.7 (C-a1), 41.7 (C-5 or C-11), 41.8 (C-11 or C5), 48.6 (C-b1), 51.3 (C-8), 53.4 (C-2), 53.6 (C-14), 108.2 (C-b6), 122.7 123.2 (C-a11, C-c11, C-a14, C-c14), 123.2 123.4 (C-a12,

aC-c12), 125.2 (C-a4 and/or C-c4), 126.9 (C-a10nd/or C-c10), 128.1 (C-a3 and/or C-c3), 128.2 (C-a8 and /or C-c8), 128.9 (C-a9 and /or C-c9), 131.2 (C-a13 and /or C-c13), 132.9 (C-a15 and /or C-c15), 141.5 (C-b7), 151.0 (C-b3), 164.2 (C-b5), 168.2 (C-3 or C-6 or C-9 or C-12), 168.3 (C-3 or C-6 or C-9 or C-12), 168.8 (C-3 or C-6 or C-9 or C-12), 172.6 (C-15).

MS (ESI) 127.2 (12%), 168.2 (7%), 235.2 (6%), 292.2 (2%), 320.2 (5%), 337.2 (3%), 389.1 (2%), 426.4 (100%, $[M + 2 H]^{2+}$), 851.4 (2%, $[M + H]^{+}$).

2. 3. Spectroscopy, ds-DNA Binding Studies

Calf thymus (ct-)

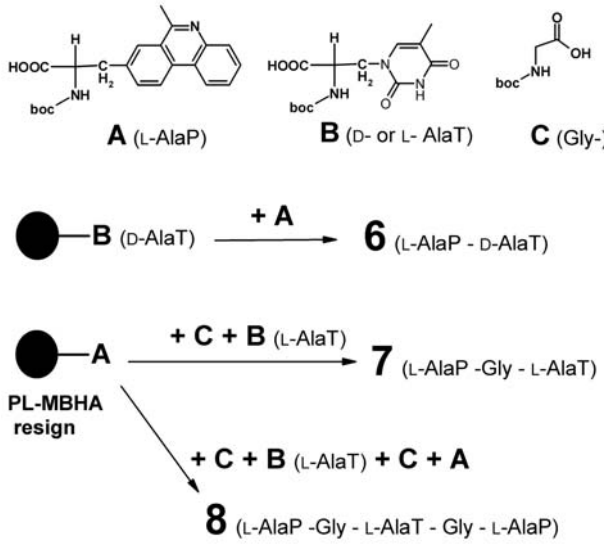
DNA was purchased from Aldrich, dissolved in Na-cacodylate buffer, I = 0.05 M, pH = 7 and additionally sonicated and filtered through a 0.45 μm filter.[18] Polynucleotide concentration was determined spectroscopically[19] as concentration of phosphates.

The electronic absorption spectra were obtained on Varian Cary 100 Bio spectrometer, CD spectra were collected on the Jasco J-815 spectrometer and fluorescence spectra were recorded on a Varian Cary Eclipse fluorimeter; all in quartz cuvettes (1 cm). The pH measurement was carried out using Mettler TOLEDO MP220 pHmeter calibrated with commercially available buffered aqueous solutions of pH standards 4.05 and 7.00. The measurements were performed in aqueous buffer solution (pH = 7.0 – buffer Na cacodylate, I = 0.05 M, pH = 5.0– buffer citric acid/NaOH, I = 0.03 M). The binding constant (K_s) and [bound**6-8**] / [DNA phosphate] ratio (n) were calculated according to the Scatchard equation by non-linear least-square fitting, giving excellent correlation coefficients (>0.999) for obtained values for K_s and n. Thermal denaturation curves for ct-DNA and its complexes with **6-8** were determined as previously describedby following the absorption change at 260 nm as a function of temperature.[18,19]The absorbance of **6-8** was subtracted from every curve, and the absorbance scale was normalized. Obtained T_m values are the midpoints of the transition curves, determined from the maximum of the first derivative or graphically by a tangent method. Given ΔT_m values were calculated subtracting T_m of the free nucleic acid from T_m of complex. Every ΔT_m value here reported was the average of at least two measurements, the error in ΔT_m is ± 0.5 °C.

3. Results and Discussion

3. 1. Synthesis and Spectroscopic Characterisation in Aqueous Medium

Analogously to recently described synthesis of peptide-bridged bis-phenanthridine derivatives,[15] novel phenanthridine-thymine conjugates (**6**, **7**) and bis-phenanthridine-thymine conjugate **8** were prepared as shown on Scheme 4 (synthetic procedures and spectroscopic characterization are given in the Experimental section). It should be noted that compound **6** differs from **7** and **8** by starting monomer (D-Ala-T) attached to the PLMBHA-resin, consequently only conjugate **6** has a free carboxylic group in proximity to thymine.



Scheme 4. A general procedure of applied solid phase synthesis.

Interestingly, solubility of **6-8** in water at neutral conditions ($c \approx 1 \times 10^{-3}$ M) is significantly higher than solubility of previously studied phenanthridine-nucleobase[11,12] and bis-phenanthridine-nucleobase conjugates,[13] most likely due to the influence of carboxylic groups present only in **6-8**. Under the experimental conditions used buffered aqueous solutions of studied compounds were stable for more days, the absorbencies were proportional to concentrations of **6-8** up to $c = 3 \times 10^{-5}$ M and reproducibility of UV/Vis spectra upon heating to 85 °C and cooling back to 25 °C was excellent, spectra before and after heating differing less than 1%. The protonation of phenanthridine nitrogen resulted in only minor changes of spectrophotometric properties of **6**-**8** solutions, according to which $pK_a \sim 6$ was estimated.[15] However, some previously studied phenanthridine analogues revealed intriguing pH-dependent interactions with DNA/RNA/nucleotides, therefore all further experiments were done at pH 7.0 and pH 5.0.[11,12,15]

Similar to previously studied bis-phenanthridine derivatives (Scheme 1)[15] the molar absorbencies (Table 1) of **6**-**8** are significantly lower than the sum of UV-Vis spectra of monomerchromophores, whereby such hypochromic effect suggests intramolecular aromatic stacking interactions between phenanthridine and thymine subunits of **6** and **7**. The hypochromic effect is particularly

pronounced for bis-phenanthridine-thymine conjugate **8** at 250 nm.

Compounds **6** and **7** exhibited fluorescence emission typical for the phenanthridinechromophore (Figure 1), and quite similar to previously studied analogues **1, 2, 4, 5**.[15] However, fluorescence spectrum of the bis-phenanthridine-thymine conjugate **8** showed additional band at 475 nm, which can be attributed to exciplexphenanthridine-thymine-phenanthridine formation, analogously to the previously studied bis-phenanthridine **3**.[15]

Table 1. Electronic absorption maxima and corresponding molar extinction coefficients in aqueous medium.[a]

	UV/Vis
	λ_{max} / nm ($\varepsilon \times 10^3$ /dm^3 mol^{-1} cm^{-1})
2[b]	250 (53.7); 332 (2.1); 349 (2.1)
6	251 (39.6); 332 (2.0); 348 (2.0)
7	252 (53.7); 334 (2.0); 349 (1.9)
8	250 (67.4); 335 (3.2); 349 (2.9)

[a] Buffer pH = 7 (sodium cacodylate buffer, I = 0.05 mol dm^{-3}).
[b] Referent compound.[15]

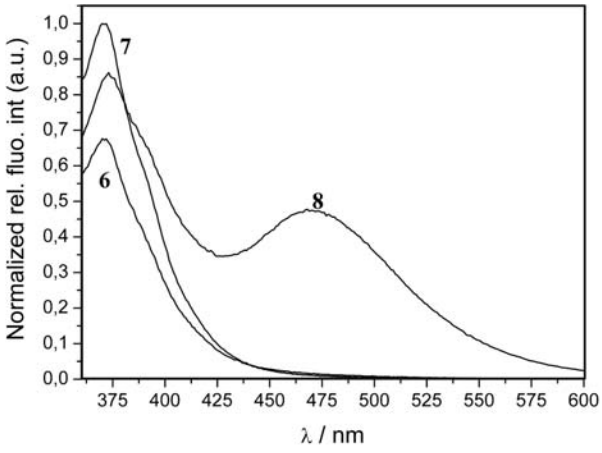


Figure 1. The fluorescence emission spectra of **6**-**8** at pH = 7.0 (buffer Na cacodylate, I= 0.05 M); c(**6**-**8**) = 5 × 10^{-6} M,λ_{exc} = 320 nm.Different parameters of the instrument were adjusted to give similar intensity of emission maxima, therefore presented fluorescence intensities cannot be directly compared.

CD spectroscopy

All studied compounds are chiral due to the stereogeniccarbon atom of substituted alanine. Compounds **6** and **7** reveal positive CD band at about 250 nm, which can be attributed to phenanthridine chromophore.[15] In comparison to **6** and **7**, the CD spectrum of **8** is of opposite sign and showed significantly stronger intensity.

Pronounced hypochromism in the UV/vis spectrum of **8** suggests strong aromatic stacking interactions, whereas fluorescence maximum at 475 nm points toward phenanthridine-thymine-phenanthridineexciplex formation (analogously to bis-phenanthridine derivative **3**).[15] Therefore, the structure of **8** in a form of self-stacked phenanthridine-thymine-phenanthridine system seems the only plausible explanation. Moreover, CD spectrum of **8** is of the opposite sign in comparison to any other analogue (**1**-**5**,[15] as well as **6** and **7**). The CD spectrum of **8** resembles closely to the CD couplets of ds-DNA (e.g. AT-DNA has also negative band at about 245 nm and positive at 270 nm),[20]which suggests helical, stacked orientation of phenanthridine-thymine-phenanthridine system, whereby mutual orientation of aromatic units mainly controls the shape of the CD spectrum. However, this is only one of the possibilities, which will be studied in more detail in the future research.The detailed NMR and crystal structure studies necessary for the exact determination of the **8** structure are in progress.

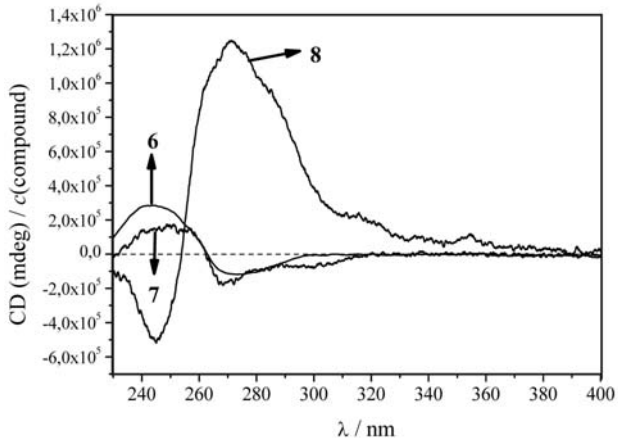


Figure 2. The CD spectra of **6**-**8** at pH = 7.0 (buffer Na cacodylate, I = 0.05 M).

3. 2. Interactions of 6-8 with Calf Thymus (ct-)DNA:

Previously studied peptide-phenanthridine derivatives showed pronounced affinity toward ds-DNA,[15] which was monitored by significant changes in their fluorescence properties. In fluorimetric titrations excitation wavelength of λ_{exc} > 300 nm was used to avoid inner filter effects caused by absorption of excitation light by added ct-DNA.Analogously, the fluorescence of **6**-**8** was strongly quenched by addition of ct-DNA (Figure 3). The experiments were performed at pH 7.0 and pH 5.0, to compare DNA binding properties of neutral and protonated (at pH 5.0 >90%)[15] phenanthridine moiety. Processing of the titration data by Scatchard analysis[21] yielded binding constants (Table 2).

Furthermore, **6**-**8** stabilized ct-DNA against thermal denaturation, rather weakly at pH 7.0 (1–5 °C) and moderately at pH 5.0 (Table 2).

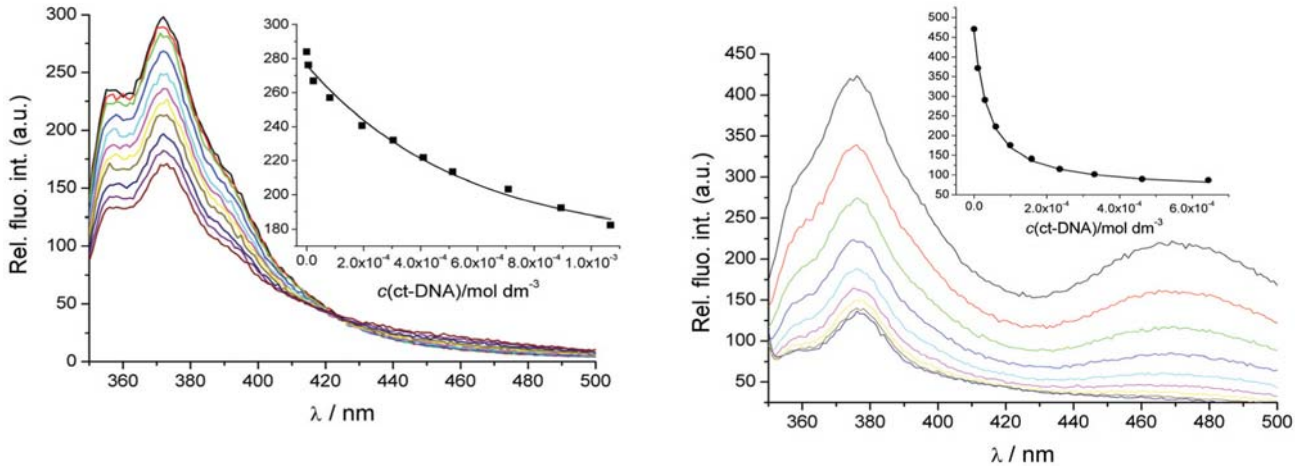


Figure 3. Fluorimetric titration of **6** (left) and **8** (right) with ct-DNA. Done at pH 5.0, I = 0.03 M, λ_{exc} = 320 nm, c(compound) = 2 × 10^{-6} M.

Table 2. Binding constants (log*Ks*)[a] calculated from the fluorimetric titrations of **1**-**8** with ct-DNA at pH = 7.0 and pH = 5.0. The [d] Δ*Tm* values (°C) of ct-DNA upon addition ofdifferent ratios [e]***r*** of **1**-**8** at pH = 5.0.

	log*Ks*		Δ*Tm*
	pH 5	**pH 7**	**pH 5**
1	5.4 [c]	4.5 [c]	4.0 [c]
2	5.4 [c]	4.7 [c]	11.1 [c]
3	6.4 [c]	b	11.3 [c]
4	6.1 [c]	6.0 [c]	18.4 [c]
5	5.9 [c]	5.4 [c]	20.2 [c]
6	5.3	4.1	1.4
7	5.3	4.5	2.0
8	5.0	5.2	14.0

[a] Done at pH 7.0(buffer Na cacodylate, I = 0.05 M) and pH 5 (buffer citric acid/NaOH, I = 0.03 M); processing of titration data by means of Scatchard equation[21] gave values of ratio n[bound**1**-**8**]/[polynucleotide] = 0.2–0.3, for easier comparison all log*Ks* values were re-calculated for the fixed n = 0.2; [b] Too small spectroscopic changes. [c] Published results.[15d] Done at pH 5, (buffer citric acid/NaOH, I = 0.03 M); error in Δ*Tm* : ± 0.5°C; [e] $\boldsymbol{r}_{[compound]\,/\,[ct\text{-}DNA]}$ = 0.3.

Affinity of **6** and **7** toward ct-DNA, as well as thermal stabilisation effects are quite similar to values obtained for previously studied mono-phenanthridine analogues **1**, **2**, thus suggesting similar binding mode (intercalation of phenanthridine moiety within DNA). Binding constant obtained for **8** is order of magnitude lower than *Ks* values of dimmeric compounds **4** and **5** and it is similar to *Ks* values obtained for **6**, **7**. However, thermal stabilisation effect of **8** is much closer to the stabilisation effects of **4** and **5** and it issignificantly stronger than observed for **6**, **7**. This discrepancy between binding affinity and thermal stabilisation effect of **8**could be explained by two phenanthridine units of **8** forming more binding interactions with ct-DNA than one phenanthridine of **6**, **7** (thus **8** yielding stronger thermal stabilisation of DNA double helix) but energetic cost of intramolecular rearrangement of **8** caused by binding to DNA lowered the value of the apparent binding constant determined by fluorimetric titration.

Circular dichroism

The CD spectra of the ligand-polynucleotide complexes can provide information on several levels.[22] Theconformation change of polynucleotide upon small molecule binding can be probed through the changes of the intrinsic polynucleotide CD spectrum. Furthermore, CD spectrum of chiral small molecules can also change upon interaction with DNA. Moreover, chromophores in small molecules could acquire an induced CD (ICD) signal when bound to DNA, from which an orientation of chromophore unit in respect to polynucleotide chiral axis could be derived, consequently giving an useful information about modes of interaction.[23,24]

Upon mixing **6** with ct-DNA, resulting CD spectra (Figure 4) significantly differed from the sum of the CD spectrum of the free DNA and CD spectrum of free **6** (the impact of the latter can be neglected due to low molar elipticity of **6** and high excess of DNA). The isoelliptic point at 265 nm pointed toward formation of one dominant type of **6**/DNA complex. An decrease of the intensity ofct-DNA CD bands at 245 and 275 nm can be attributed to partial unwinding of double helix caused by the binding of **6**, and new band at 261 nm could be attributed to ICD band of **6-**phenanthridine.Intriguingly, addition of **7** resulted in only minor decrease of the DNA CD band at 275 nm, while the rest of the DNA CD spectrum remained basically the same (results not shown).

The isoelliptic point at 267 nm pointed toward formation of one dominant type of **8**/DNA complex (Figure 4, right). Although CD spectra of the free ct-DNA and free **8** closely resemble by sign and the shape of CD bands, the mixing of **8** and ct-DNA didn't sum up their CD spectra but instead resulted in radical changes in the shape of CD bands, whereby also the sign of both CD

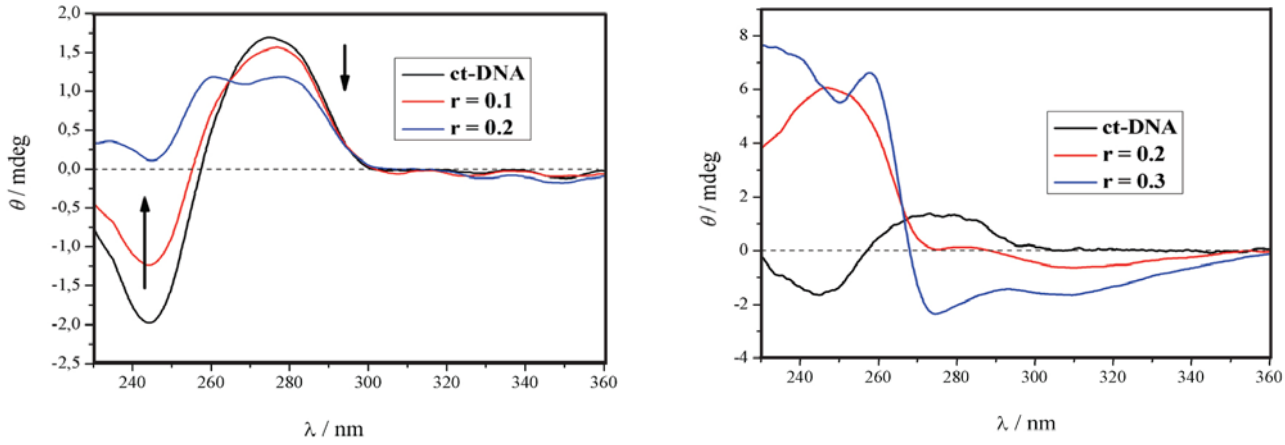


Figure 4. CD titration of ct-DNAwith **6** (left) and **8** (right), *c*(ct-DNA) = 2 × 10^{-5} M; ratio r = [compd.] / [ct-DNA]. Done at pH 5.0, (buffer citric acid/NaOH, *I* = 0.03 M). For the CD spectrum of **8**/ct-DNA; r = 0.3 at λ < 250 nm total absorbance of the sample was Abs>2, thus CD curve for that range is not accurate.

bands was inverted. The observed inversion of the sign of CD bands could be explained in two ways: a) DNA double helix was inverted from the right-handed to left-handed; or b) the phenanthridines of **8** upon binding to DNA revealed strong ICD bands (positive < 270 nm and negative > 270 nm), which were superimposed to the CD bands of DNA. The possibility a) is not likely to happen for the mixed basepairsct-DNA, since left-handed DNA's consist mostly of G-C basepairs. Moreover, new maximum at 260 nm closely resembles to maximum attributed to ICD spectrum of **6** (Figure 4, left). Thus, observed results could be interpreted as DNA-driven disintegration of self-stacked structure of **8** followed by incorporation of phenanthridine subunits into chiral surrounding of DNA double helix, giving rise to observed ICD bands.

4. Conclusions

Novel phenanthridine-thymine conjugates **6**-**8**were prepared by solid phase peptide synthesis, which proved to be significantly simpler and faster than previously used methodologies for preparation of aryl-nucleobase conjugates.[6,7,12,13] Moreover, it allowed easy and fast tuning of compound properties by choosing the order of added amino-acid monomers. In aqueous medium **6**-**8** are mostly present in intramolecularly stacked form, whereby stacked phenanthridine-thymine-phenanthridine system of **8** revealed characteristic excimeric fluorescence band at 475 nm and very specific CD spectrum.

The binding properties of **6**, **7** to ct-DNA were similar to previously studied analogues **1**, **2**, pointing out that attached thymine (**6**, **7**) does not alter significantly the ability of phenanthridine to intercalate between DNA basepairs. The ICD bands observed upon binding of **6** to ct-DNA but not for 7 stressed the importance of amino-acid sequence attached to phenanthridine moiety, whereby small and neutral amino group of **6** allowed much easier accommodation of phenanthridine chromophore inside DNA in comparison to sterically demanding –COO^- moiety of **7**, which in addition could yield repulsive interactions toward DNA phosphates. This finding could find use in the design of new generations of peptide-based analogues, allowing fine tuning of attractive and repulsive interactions with ds-DNA.

Two phenanthridine units of **8** formed more binding interactions with ct-DNA than one phenanthridine of **6**, **7** (thus **8** yielding stronger thermal stabilisation of DNA double helix) but energetic cost of intramolecular rearrangement of **8** caused by binding to DNA lowered the value of the apparent binding constant determined by fluorimetric titration. However, thymine attached between phenanthridines didn't abolish efficient binding of **8** to double stranded DNA, although from the observed experimental results it is not clear whether one of both phenanthridine units of **8** intercalate into DNA.

Presented results encourage further studies of interactions of **6**-**8** with single stranded sequences of DNA/RNA,[11,12] as well as double stranded DNA with abasic sites containing complementary nucleobase (adenine).[7] In addition, preliminary results of low antiproliferative activity of here presented compounds on a human cell lines support their applicability as selective blockers of the repair of abasic DNA sites, caused by classical antitumour therapy.

5. Acknowledgment

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6.References

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Povzetek

Niz novih peptidnih konjugatov fenantridin-nukleotidna baza je bil pripravljen s sintezo na trdnem nosilcu, ki omogoča enostavno in hitro uravnavanje strukturnih lastnosti spojin. Lastnosti spojin proučevanih v vodnem mediju kažejo na tvorbo intramolekularno naloženih (»stacked«) struktur. Takšna struktura v sistemu fenantridin-timin-fenantridin kaže značilen fluorescenčni spekter in zelo specifičen CD spekter. Proučevane spojine interagirajo z dvoverižno DNA z interkalacijo, pri čemer na vezanje v manjši meri vplivata tudi vanje vključen timin in aminokislinsko zaporedje peptidne verige.