Journal of Peptide Science
J. Pept. Sci. 2006; **12**: 472–480
Published online 27 February 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.752

JOURNAL OF PEPTIDE SCIENCE

New peptide conjugates with 9-aminoacridine: synthesis and binding to DNA

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Received 6 December 2005; *Revised November 2005*; *Accepted 6 January 2006*

Abstract: New peptides – 9-aminoacridine conjugates with an ethylene diamine linker – have been synthesized (both solution and solid phase methods were used) and their interactions with DNA have been studied. The affinity of H-Phe-Gln-Gly-Ile_2-$NHCH_2CH_2NH$-Acr conjugate and of its extended analogue containing 6-aminohexanoic acid to DNA were lower than that of a standard H-Gly-$NHCH_2CH_2NH$-Acr conjugate. The results fit well into our concept of peptide conjugates with lowered binding activity to DNA, which could be capable of unlimited extravascular distribution. Moreover, new structures could be potentially useful as the mild tuners of DNA interaction with strong bis-acridine binders. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptide-9-aminoacridine conjugates; mild intercalators into DNA; solution synthesis; polymer supported synthesis; tuners of DNA interaction with strong binders

INTRODUCTION

The tight binding of some drugs to DNA may correlate with their cytotoxicity. However, it can also severely limit their extravascular distribution, since diffusion in the body is driven by free drug concentration. The effective diffusion coefficient D_{ef}, which determines the rate of extravascular distribution of the drug, is significantly lowered by its strong binding to DNA, expressed by the binding constant K_{DNA}. The upper limit of K_{DNA}, approximately 10^4 M^{-1}, is assumed to enable sufficiently rapid diffusion time on the pharmacological timescale, suggesting that even moderate values of DNA binding (10^5–10^6 M^{-1}) can limit diffusion.

These effects of the DNA-binding on drug diffusion have been shown in several tumors in which poor distribution of the compounds tightly bound to the DNA was observed. On the other hand, a weaker drug-binding to the DNA exhibited higher free–drug diffusion coefficients. Thus the concept of 'minimal intercalators' was proposed to include the requirements of the mandatory intercalative binding mode together with the lowest possible level of binding, and a search was carried out for structures that fulfilled these requirements [1–3].

Recently, 9-aminoacridine was reported to intercalate into DNA [4–6] with a K_{DNA} of approximately 10^5 M^{-1}. Also, some peptide conjugates with this molecule were synthesized and shown to exert even stronger binding activity [7,8].

Incorporation of the acridine chromophore imparts a planar structure to molecules, which allows them to bind DNA by stacking between base pairs [2]. As a result a high affinity for DNA is observed, which is generally considered a biological target for the acridine anticancer agents. The noncovalent interaction associated with ligands binding to DNA, such as electrostatic, $\pi \rightarrow \pi$ stacking, and hydrogen bonding, involves changes of both the DNA and the drug molecules to accommodate a complex. The DNA duplex changes result in an altered thermodynamic stability and biochemical properties of the DNA [9,10]. In the case of cationic intercalators, additional counterions are released during this event through a process known as polyelectrolyte effect [11,12]. In addition to the energy of the intercalation complex, some chemical substituents associated with the chromophore also provide additional contributions to the DNA-binding energy. This process is enhanced by electrostatic and hydrophobic interactions of the functional groups residing on the DNA within the major/or minor grooves at the intercalation site [13].

Keeping in mind the above findings, we decided to design new 9-aminoacridine–DNA binders with low affinity to nucleic acid. To reduce DNA-binding constant of 9-aminoacridine, we suggest connecting two DNA-binders with two different binding sites in the molecule of the nucleic acid. We could illustrate this intention by using a parable about the molecules behaving like two stiff-necked boys who want one

Abbreviations: Acr, acridin-9-yl; εAhx, ε-aminohexanoic acid; AAA, amino acid analysis; CT-DNA, calf thymus DNA; Et_2O, diethyl ether; EtOAc, ethyl acetate; MeOH, methanol; TOTU, *O*-[(ethoxycarbonyl) cyanomethylenamino]-*N*, *N*, *N*′, *N*′-tetramethyluronium tetrafluoroborate. The nomenclature and symbols of amino acids as well as the abbreviations follow 'A revised guide to abbreviations in peptide science and a plea for conformity' (J. Peptide Sci. 2003; **9:** 1–8).

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toy. If both pull it, neither can get it. The same behavior is expected for the molecule with two DNA-binders tied in proximity. Since the 9-aminoacridine is supposed to be a *gc*-DNA-binder [14], we have looked for a peptide moiety, which is an *at*-DNA-binder. Recently, Sasaki and coworkers [15] have found that a pentapeptide H-Phe-Gln-Gly-Ile$_2$-OH binds selectively to oligonucleotides (Figure 1) with relatively high DNA-binding constants. Since conjugates of this peptide with 9-aminoacridine have not been described yet, we decided to prepare similar peptide molecules conjugated to 9-aminoacridine. We intended to determine their binding constants, and subsequently to assess the effect of peptide-acridine conjugation on the efficiency of binding to DNA. To avoid repulsion with the negatively charged DNA backbone, we changed the *C*-terminal carboxyl group for carboxamide in the corresponding molecules.

MATERIALS AND METHODS

General

The peptides were synthesized either on polymeric support by the Fmoc/*t*Bu strategy, or in solution using the Boc protection. The course of the coupling reaction was monitored by the Kaiser [16] and bromophenol blue [17] tests. Samples for the AAA were hydrolyzed with 6 M HCl containing 3% phenol at 110 °C for 20 h (unless stated otherwise). The amino acid analyses were performed on a Biochrom 20 instrument (Pharmacia, Sweden). Molecular weights of peptide fragments were determined using mass spectroscopy with FAB technique (Micromass, Manchester, UK). For HPLC, a Spectra Physics instrument with an SP 8800 pump, an SP 4290 integrator, and a Thermo Separation Products Spectra 100 UV detector were used. The peptides were purified by semipreparative HPLC on a 25 cm × 1 cm column, 10 μm Vydac RP-18 or RP-8 (The Separations Group, Hesperia CA, USA), flow rate 3 ml/min, detection at 220 nm using gradient 0–100% CH_3CN in 0.05% aqueous TFA over 60 min. The analytical HPLC was carried out on a 25 × 0.4 cm column, 5 μm LiChrospher WP-300 RP-18 (Merck, Darmstadt, Germany), flow rate 1 ml/min, detection at 220 nm, using 0–100% gradient of CH_3CN in 0.05% aqueous TFA over 40 (grad40) or 60 min (grad60). ^{1}H NMR spectra were measured on a Varian UNITY-200 or a Bruker AVANCE-400 spectrometers.

Figure 1 Targeted oligonucleotide that strongly binds the peptide H-Phe-Gln-Gly-Ile$_2$-OH.

Boc-Phe-Gln-OH (1)

A solution of glutamine (0.37 g, 2.51 mmol) in a DIEA : water mixture 1 : 7 (4 ml) and Boc-Phe-OSu (1 g, 2.76 mmol) in dioxane (6 ml) was stirred overnight. To the suspension, 1% solution of DIEA in water (20 ml) was added and the solution was washed three times with ethyl acetate (EtOAc) (50 ml). The aqueous layer was cooled and adjusted to pH 2 with HCl (1 : 1). The aqueous layer was immediately washed with EtOAc (2 × 50 ml) and the combined organic washing was dried with anhydrous Na_2SO_4 and evaporated. Compound **1**, after crystallization from an EtOAc-hexane mixture was obtained in a yield of 0.89 g (2.26 mmol) with m.p. 113–116 °C (Ref. 18 112–114 °C). HPLC (grad60) peak retention time was 31.5 min. For $C_{19}H_{27}N_3O_6$ (393.19) found, *m/z*: 394 ($M + H^+$), 416 ($M + Na^+$) by FAB-MS.

N-(Benzyloxycarbonyl)-ethane-1,2-diamine (2)

The procedure similar to the preparation of propane-1,3-diamine analogue described elsewhere was adopted [19]. A solution of Z-OSu (4.2 g; 18 mmol) in $CHCl_3$ (30 ml) was added drop wise to a stirred solution of ethylene diamine (32 ml; 480 mmol) in $CHCl_3$ (100 ml) at 7 °C within 2 h. After removal of the cooling bath, the reaction mixture was stirred overnight. The organic layer was washed with 1 M $NaHCO_3$ (2×), with a saturated solution of Na_2SO_4, dried with anhydrous Na_2SO_4, and evaporated *in vacuo*. Compound **2** was obtained in a yield of 3.2 g (16 mmol) as a low melting white solid with m.p. 34–37 °C (Ref. 20 157–159 °C for corresponding hydrochloride) and was used without further purification.

N-(Benzyloxycarbonyl)-*N'*-(acridin-9-yl)ethane-1,2-diamine hydrochloride (3)

9-Chloroacridine [21], prepared from *N*-phenylanthranilic acid (4 g, 29 mmol), was treated with compound **2** (3.2 g, 16 mmol) according to the procedure described for preparation of the propane-1,3-diamine analogue [22] to obtain a compound **3** in a yield of 3.6 g (9 mmol) with m.p. 183–185 °C. TLC in a $CHCl_3$–methanol (MeOH)–Et_3N (15 : 2 : 1) mixture R_f = 0.53; HPLC (grad40) peak retention time was 18.8 min. For $C_{23}H_{22}ClN_3O_2$ (407.7) calculated: 67.73% C, 5.44% H, 10.30% N, 8.69% Cl; found: 67.52% C, 5.50% H, 10.21% N, 8.73% Cl. For $C_{23}H_{21}N_3O_2$ (371.2) found *m/z*: 372.2 ($M + H^+$) by FAB-MS. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.56 (m, 2H, CONH-CH_2); 4.21 (m, 2H, acridin-9-yl Acr-NH-CH_2); 4.97 (s, 2H, Ph-CH_2); 7.26 (m, 5H, Ph-H); 7.53 (m, 2H, Acr-H); 7.71 (t, 0.8 H, J = 5.5, CO-NH); 7.98 (d, 4 H, J = 6.0, Acr-H); 8.62 (d, 2H, J = 8.6, Acr-H); 9.92 (b, 0.6 H, Acr-NH); 14.25 (b, 0.5 H, H-N^+ (Acr)).

N-(Acridin-9-yl)ethane-1,2-diamine dihydrobromide (4)

Compound **3** (0.4 g, 0.98 mmol) was suspended in a 4.1 M HBr-AcOH solution (20 ml) and sonicated for 1 h. The mixture was poured into dry diethyl ether (Et_2O) (80 ml) and a yellow precipitate was decanted, triturated with additional Et_2O (80 ml) and filtrated by suction. The precipitate was washed with Et_2O (4×) on the filter and hygroscopic dihydrobromide was immediately dried in a desiccator over P_2O_5 linked to vacuum in a yield of 0.34 g (0.86 mmol) of compound **4**, which

decomposed at 280 °C. HPLC (grad40) peak retention time was 10.4 min. For $C_{15}H_{17}Br_2N_3$ (399.1) calculated: 45.14% C, 4.29% H, 10.53% N, 40.04% Br; found: 45.42% C, 4.50% H, 10.35% N, 39.70% Br. For $C_{15}H_{15}N_3$ (237.15) found, *m/z*: 238.0 ($M + H^+$) by FAB-MS. ^1H NMR (400 MHz, DMSO-d_6): δ 3.44 (m, 2H, N^+H_3-CH_2); 4.41 (m, 2H, Acr-NH-CH_2); 7.59 (t, 2H, $J = 8$, Acr-H); 7.95 (m, 6H, Acr-H + N^+ H_3); 8.62 (d, 2H, $J = 8.6$, Acr-H); 9.70 (b, 0.5 H, Acr-NH); 13.66 (b, 0.5 H, H-N^+ (Acr)).

Peptide derivatives of *N*-(acridin-9-yl)ethane-1, 2-diamine (5–17)

To a solution of Z- or Boc-amino acid or Boc-peptide (0.5 mmol), PyBOP (0.26 g; 0.5 mmol) and HOBt (0.067 g; 0.5 mmol) in DMF (4 ml), the DIEA (0.085 ml; 0.5 mmol) was added under stirring.

After 5 min, *N*-(acridin-9-yl)ethane-1,2-diamine dihydrobromide (**4**), (0.21 g, 0.5 mmol) or corresponding *N*-(acridin-9-yl)-*N'* -(aminoacyl)ethane-1,2-diamine trifluoroacetate or *N*-(acridin-9-yl)-*N'* -peptidylethane-1,2-diamine trifluoroacetate (0.5 mmol) and another portion of DIEA (0.17 ml; 1 mmol) were added to the reaction mixture. The reaction course was monitored by paper electrophoresis as disappearance of the amino component. When the reaction was complete, the solvent was evaporated, the residue dissolved in EtOAc (25 ml) and the solution washed with 1 M $NaHCO_3$ (3 ml × 20 ml). The organic layer was dried with anhydrous Na_2SO_4 and, after filtration and evaporation, the product was obtained in the yield 70–80%. Before each synthetic step, the Boc deprotection was carried out with neat TFA for 1 h at room temperature. After TFA was evaporated, the residue was washed with dry Et_2O and finally dried in a desiccator. The corresponding TFA salts were obtained in yields of *ca* 90%. In compound **12,** the Z deprotection was carried out with 4.1 M HBr in AcOH (10 ml) for 30 min according to the procedure described in the preparation of compound **4**. Analytical parameters of compounds **5**–**17** are shown in Tables 1 and 2.

Boc-Gln-Gly-OBzl (18)

To a stirred solution of H-Gly-OBzl hydrochloride (2 g; 10 mmol), Boc-Gln-OH (2.7 g; 11 mmol) and HOBt (1.68 g; 11 mmol) in DMF (10 ml), DCC (2.3 g; 11 mmol) was added at −15 °C and the reaction mixture was stirred for 30 min. Then DIEA (1.7 ml; 10 mmol) was poured to the suspension and the reaction mixture was left standing overnight. The chilled suspension was filtered off and DMF evaporated. The residue was dissolved in EtOAc and washed with 1 M $NaHCO_3$ (3 × 20 ml), brine (1 × 20 ml), a 20% citric acid solution (3 × 20 ml) and brine (1 × 20 ml). The organic layer was dried with anhydrous $MgSO_4$ and evaporated. The product **18** was obtained in a yield of 3.15 g (8 mmol) with m.p. 107–109 °C (Ref. 23 108–110 °C). For $C_{19}H_{27}N_3O_6$ (393.19) found *m/z*: 394.0 ($M + H^+$) by FAB-MS.

Boc-Phe-Gln-Gly-OBzl (19)

Compound **18** was dissolved in a freshly prepared mixture of TFA and anisole (20 : 1). After 50 min of occasional stirring, the reaction mixture was evaporated to dryness and the residue was triturated with dry Et_2O. The peptide was crystallized from EtOAc as a TFA salt. This salt (3.21 g; 7.9 mmol), Boc-Phe-OSu (2.9 g; 7.9 mmol), HOBt (0.52 g; 7.9 mmol) and DIEA (1.33 ml; 7.8 mmol) in DMF (10 ml) were stirred overnight. The solvent was evaporated to dryness, the residue was dissolved in EtOAc and the solution washed with 1 M $NaHCO_3$, brine, a

Table 1 Analytical parameters of 9-aminoacridine peptide derivatives **5**–**10**

Compound (No.)	Formula FAB MS[b]	HPLC RT (min)[c]	Electrophoresis[a] $E^{Gly}_{2.4}$	$E^{His}_{2.4}$	$E^{His}_{5.7}$
Boc-Gly-X[d] (**5**)	$C_{22}H_{26}N_4O_3$ 394.2/395.1	—	1.0	0.64	0.72
TFA.H-Gly-X[d] (**6**)	$C_{17}H_{18}N_4O$ 294.2/295.1	15.8	1.84	1.13	1.25
Boc-Ile-X[d] (**7**)	$C_{28}H_{34}N_4O_3$ 450.3/451.3	36.0	1.84	1.13	1.15
Boc-Ile_2-X[d] (**8**)	$C_{32}H_{45}N_5O_4$ 563.3/564.3	40.5	1.83	1.14	1.17
Boc-Gly-Ile_2-X[d] (**9**)	$C_{34}H_{48}N_6O_5$ 620.3/621.3	40.3	1.81	1.12	1.13
Boc-Phe-Gln-Gly-Ile_2-X[d] (**10**)	$C_{48}H_{65}N_9O_8$ 895.5/896.4	43.1	1.77	1.07	1.08

[a] Electrophoresis was carried out after removal of the corresponding protecting groups (with the exception of compound **5**), on Whatman 3 MM paper for 45 min at a potential drop of 20 V/cm, in 1 M acetic acid (pH 2.4) and pyridine-acetate (pH 5.7) buffer with ninhydrine detection.
[b] Molecular weights were determined using mass spectroscopy with FAB technique (Micromass, Manchester, England).
[c] Analytical HPLC was performed on a 25 cm × 0.4 cm column, 5 μm LiChrospher WP-300 RP-18 (Merck, Darmstadt, Germany), flow rate 1 ml/min, detection at 220 nm, using 0–100% gradient of CH_3CN in 0.05% aqueous TFA in 60 min. Spectra Physics instrument with an SP 8800 pump, an SP 4292 integrator and a Thermo Separation Products Spectra 100 UV detector was used.
[d] X = NH–CH_2–CH_2–NH-Acr.

Table 2 Analytical parameters of 9-aminoacridine peptide derivatives **11**–**17**

Compound (No.)	Formula FAB MS[b]	HPLC RT (min)[c]	Amino acid analysis[a]				
			Phe	Glu	Gly	Ile	εAhx[d]
TFA. H-Phe-Gln-Gly- Ile_2-X[e] (**11**)	$C_{43}H_{57}O_6N_9$ 795.4/796,3	32.7	0.97	0.94	1.00	1.73	—
Z-εAhx-X[e] (**12**)	$C_{29}H_{32}O_3N_4$ 484.3/485.2	35.6	—	—	n.d.	—	—
Boc-Ile-εAhx-X[e] (**13**)	$C_{32}H_{45}N_5O_4$ 563.5/564.4	39.7	—	—	—	0.95	1.00
Boc-Ile_2-εAhx-X[e] (**14**)	$C_{38}H_{56}N_6O_5$ 676.4/677.4	35.8	—	—	—	1.92	1.00
Boc-Gly-Ile_2-εAhx-X[e] (**15**)	$C_{40}H_{59}N_7O_6$ 733.5/734.4	35.1	—	—	1.03	1.89	1.00
Boc-Phe-Gln-Gly-Ile_2-εAhx-X[e] (**16**)	$C_{54}H_{76}N_{10}O_9$ 1008.6/1009.3	39.3	1.00	0.89	1.00	1.60	1.09
TFA.H-Phe-Gln-Gly-Ile_2-εAhx-X[e] (**17**)	$C_{49}H_{68}O_7N_{10}$ 908.5/909.3	26.9	—	—	n.d.	—	—

[a] Samples for amino acid analysis were hydrolyzed with 6 M HCl containing 3% of phenol at 110 °C for 20 h. Analyses were performed on a Biochrom 20 instrument (Pharmacia, Sweden).
[b] Molecular weights were determined using mass spectroscopy with FAB technique (Micromass, Manchester, UK).
[c] Analytical HPLC was performed on a 25 × 0.4 cm column, 5 μm LiChrospher WP-300 RP-18 (Merck, Darmstadt, Germany), flow rate 1 ml/min, detection at 220 nm, using 0–100% gradient of CH_3CN in 0.05% aqueous TFA in 60 min. Spectra Physics instrument with an SP 8800 pump, an SP 4292 integrator and a Thermo Separation Products Spectra 100 UV detector was used.
[d] ε-Aminohexanoic acid.
[e] X = NH–CH_2–CH_2–NH-Acr.

20% citric acid solution and brine. The organic layer was dried with anhydrous $MgSO_4$ and evaporated. The product **19** was obtained in a yield of 2.95 g (5.5 mmol) with m.p. 115–118 °C. For $C_{28}H_{36}N_4O_7$ (540.6) found *m/z*: 541.5 ($M + H^+$) by FAB-MS. AAA: Glu (1.0), Gly (1.0), Phe (1.2). ^{1}H NMR (200 MHz, DMSO-d_6): δ 1.32 (s, 9 H, (Boc)); 1.32–2.50 (m, 2H, β-H_2 (Gln)); 2.50–3.33 (m, 4 H, γ-H_2 (Gln) and β-H_2 (Phe)); 3.92 (m, 2H, α-H_2 (Gly)); 4.15 (m, 1H, α-H (Gln)); 4.30 (m, α-H (Phe)); 5.13 (s, 2H, CH_2Ph); 6.79 (s, 2H, NH_2 (Gln)); 6.96 (d, 1H, *J* = 8.2, NH (Phe)); 7.25–7.38 (m, 10 H, 2 × C_6H_5 (Phe + Ph)); 8.05 (d, 1H, *J* = 8.2, NH (Gln)); 8.44 (t, 1H, *J* = 8.1, NH (Gly)).

Boc-Phe-Gln-Gly-OH (20)

The Bzl group of compound **19** (0.54 g, 1 mmol) was removed by hydrogenolysis on Pd/charcoal in MeOH at 40 °C for 5 h. After filtering the catalyst and evaporation, the compound **20** was obtained in a yield of 0.4 g (0.89 mmol) with m.p. 121–123 °C. For $C_{21}H_{30}N_4O_7$ (450.5) found *m/z*: 451.5 ($M + H^+$) by FAB-MS. ^{1}H NMR (200 MHz, DMSO-d_6) : δ 1.33 (s, 9 H, (Boc)); 1.32–2.50 (m, 2H, β-H_2 (Gln)); 2.50–3.35 (m, 4 H, γ-H_2 (Gln) and β-H_2 (Phe)); 3.94 (m, 2H, α-H_2 (Gly)); 4.14 (m, 1H, α-H (Gln)); 4.31 (m, 1H, α-H (Phe)); 6.81 (s, 2H, NH_2 (Gln)); 6.94 (d, 1H, *J* = 8.2, NH (Phe)), 7.23–7.36 (m, 5 H, C_6H_5 (Phe)); 8.05 (d, 1H, *J* = 8.2, NH (Gln)); 8.44 (t, 1H, *J* = 8.1, NH (Gly)); 12.1 (b, 0.5 H, COOH (Gly)).

Fmoc-Ile-O-ClTrt-Resin (21)

To 2-chlorotrityl chloride-polystyrene resin (1.5 g, 1.26 mmol. g^{-1}, 1.89 mmol) swollen in THF, a solution of Fmoc-Ile-OH (0.5 g, 1.41 mmol) and DIEA (0.2 ml, 1.17 mmol) in THF (10 ml) was added and the reaction mixture was stirred for 10 min. Then, another portion of DIEA (0.4 ml, 2.35 mmol) was added and the mixture was stirred for another 1 h. Capping with MeOH (1.2 ml) was carried out within 10 min and the resin was washed with THF (3×), DMF (2×), iPrOH (2×), DMF (2×), iPrOH (2×), MeOH (2×) and Et_2O (2×). After drying, resin **21** loaded with amino acid (substitution 0.21 mmol.g^{-1} as determined by AAA) was obtained in the yield 1.6 g.

Fmoc-Phe-Gln(Trt)-Gly-Ile_2-OH (22)

This peptide sequence was assembled on resin **21** (0.5 g; 0.1 mmol). The Fmoc protecting group was removed by the treatment with 5% piperidine in DMF (30 ml; 10 min) and 20% piperidine in DMF (30 ml; 30 min), which was also used in the following deprotection steps, to obtain H-Ile-O-ClTrt-Resin. This resin was gradually acylated with 0.35 mmol of Fmoc-Ile-OH (123 mg), Fmoc-Gly-OH (104 mg), Fmoc-Gln(Trt)-OH (214 mg) and Fmoc-Phe-OH (136 mg) in the presence of HOBt (54 mg; 0.35 mmol) and DIC (62 μl; 0.4 mmol) as the coupling reagents in DMF (30 ml). The progress of the couplings was monitored by the Kaiser [16] and bromophenol blue [17] tests. The peptide-resin prepared (0.6 g) was suspended in an AcOH–DCM–TFE (23 : 71 : 6) mixture (10 ml) and stirred for 30 min. The solution was collected and detachment of the peptide from the resin was repeated twice. Combined solutions were evaporated and covered with water. A white precipitate was collected and dried by lyophilization overnight. The protected peptide **22** was obtained in a yield of 0.16 g (0.15 mmol). HPLC (grad 50–100% CH_3CN, 30 min) peak

retention time was 27.7 min. AAA: Gly (1.0), Gln (1.0), Ile (1.5), Phe (0.7). For $C_{62}H_{68}N_6O_9$ (1040.5) found m/z: 1041.7 ($M + H^+$), 1063.7 ($M + Na^+$) by FAB-MS.

Fmoc-Phe-Gln(Trt)-Gly-Ile$_2$-NHCH$_2$CH$_2$NH-Acr (23) (by the PyBOP Method)

To the compound **22** (2 mg; 1.92 μmol), HOBt (0.26 mg; 1.92 μmol), PyBOP (1.04 mg; 2 μmol) and DIEA (1.7 μl; 10 μmol) in DMF (40 μl) were added and after 2 min stirring, also compound **4** (0.77 mg; 1.92 μmol). The reaction mixture was stirred overnight. The solvent was evaporated and diastereoisomers **23** (Table 3) were separated using HPLC. HPLC (grad 40) peak retention times were 24.9 min and 25.2 min. A sample for MS determination was treated with 25% diisopropylamine in DMF for 30 min and after evaporation to dryness treated with a 90% TFA/anisole mixture for 2 h. For $C_{43}H_{57}N_9O_6$ (795.44) found m/z: 796 ($M + H^+$) by FAB-MS.

Fmoc-Phe-Gln(Trt)-Gly-Ile$_2$-NHCH$_2$CH$_2$NH-Acr (23) (by the TOTU Method)

To the compound **22** (2 mg; 1.92 μmol), TOTU (0.66 mg; 2 μmol) and DIEA (1.12 μl; 6.6 μmol) in DMF (100 μl) were added and after 2 min stirring, also compound **4** (0.92 mg; 2.3 μmol). The reaction mixture of **23** was stirred for 5 h and the solvent was evaporated. The residue was dissolved in EtOAc, the solution was washed twice with 1 M $NaHCO_3$ and dried with anhydrous Na_2SO_4. After evaporation of the solvent and triturating the residue with dry Et_2O, a mixture of isomers (Table 3) was obtained, which was characterized by HPLC and MS. HPLC (grad 40) peak retention times were 24.8 min (28.2%) and 25.1 min (60.5%). A sample for MS was treated with 25% diisopropylamine in DMF for 30 min and after evaporation to dryness, with a 90% TFA/anisole mixture for 2 h. For $C_{43}H_{57}N_9O_6$ (795.44) found m/z : 796 ($M + H^+$) by FAB-MS).

Table 3 Comparison of epimerization in segment coupling in the synthesis of H-Phe-Gln-Gly-Ile$_2$-NHCH$_2$CH$_2$NH-Acr

Coupling site[a]	Coupling agents	Base/solvent	HPLC yield %	d.e.(%)[b]
I-Acr	PyBOP/HOBt	DIEA/DMF	27	72
I-Acr	TOTU	DIEA/DMF	61	68
Q-G	PyBOP/HOBt	DIEA/DMF	62	77
G-I	PyBOP/HOBt	DIEA/DMF	85	99

[a] Three different sites were employed in the formation of corresponding linkage:
Ile-NHCH$_2$ (I-Acr), Gln-Gly (Q-G) and Gly-Ile (G-I).
[b] d.e.,diastereoisomeric excess.

CT-DNA Binding

Partitioning between two aqueous phases. A two-phase stock solution was prepared by dissolving PEG 6000 (0.8 g), dextran 500 000 (1.2 g), sodium acetate trihydrate (49 mg) and acetic acid (95 μl) in water (18 ml) under continuous stirring and heating in a water bath (60 °C). This stock solution was immediately distributed into tubes and allowed to cool to 4 °C. The working volume was either 7 ml for fluorescence measurements or 1 ml for visualization. Stock solutions of 9-aminoacridine compounds and/or CT-DNA were added in appropriate amounts to each vial (tube). Calibration was carried out with variable total concentration of acridine component without DNA (Figure 2). Fluorescence emission spectra of both phases were recorded (excitation wavelength 412 nm). The binding constants (Table 4) were determined by DNA titration at the same total concentration of the acridine component.

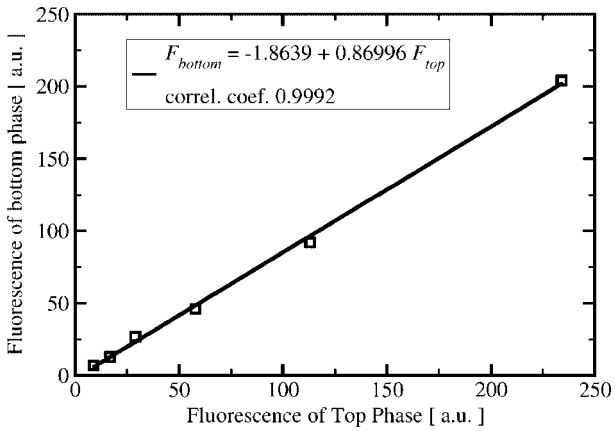

Figure 2 Calibration of H-Phe-Gln-Gly-Ile$_2$-NHCH$_2$CH$_2$NH-Acr fluorescence in both the phases. The plot displays how the fluorescence of the bottom phase depends on the fluorescence of the top phase.

Table 4 CT-DNA binding constants of H-Phe-Gln-Gly-Ile$_2$ compounds and Acr standard

Ligand	K (K_{Hill}) [L.mol^{-1}]	References
NH$_2$-Acr	(1×10^5)[a]	24
H-Gly-NHCH$_2$CH$_2$NH-Acr	(1.7×10^5)[a] (1.1×10^5) [b]	exp.
H-Phe-Gln-Gly-Ile$_2$-NHCH$_2$CH$_2$NH-Acr	(5.4×10^4)[a] (1.3×10^5) [b]	exp.
H-Phe-Gln-Gly-Ile$_2$-εAhx-NHCH$_2$CH$_2$NH-Acr[c]	(4.4×10^4)[a] (9.8×10^4) [b]	exp.

[a] Fitted with a curve for model of noncooperative one site binding – comparable to Scatchard plot.
[b] Fitted with a curve for model of cooperative one site binding – Hill model.
[c] εAhx, ε-Aminohexanoic acid.

Table 5 Melting temperatures of targeted oligonucleotide (Figure 1) in the presence of specified ligand

Ligand	T_m [°C]
None	45.05
NH_2-Acr	44.06
H-Phe-Gln-Gly-Ile$_2$-$NHCH_2CH_2NH$-Acr	45.09
H-Phe-Gln-Gly-Ile$_2$-εAhx-$NHCH_2CH_2NH$-Acr[a]	45.06

[a] εAhx, ε-Aminohexanoic acid.

Oligonucleotide Melting

The targeted oligonucleotide (1 μmol l^{-1}) (Figure 1), with or without the acridine compound (1 μmol l^{-1}) in 1 ml of buffer was heated in a Varian Bio100 spectrophotometer with continual reading of absorbance at 260 nm. The melting points (Table 5) were determined by differentiation of melting curves as the temperature at the maximum of the obtained curve.

RESULTS AND DISCUSSION

First, investigation of a suitable route for synthesis of the peptide conjugates H-Phe-Gln-Gly-Ile$_2$-$NHCH_2CH_2NH$-Acr and its analogue with 6-aminohexanoic acid as the linker was performed. We decided to start the synthesis from *N*-(acridin-9-yl)ethane-1,2-diamine, which was acylated with Boc-amino acid. Its carboxyl was activated with PyBOP [25,26], which assures fast coupling. The first stepwise synthesis was carried out in solution. However, this route possesses major drawbacks such as poor yield based on the first building block, and a very long time is required to obtain the target substance. The advantages of this method, however, consisted in obtaining a nonepimerized peptide, which could be readily purified by HPLC in a relatively high yield. According to the second route, preparation of the protected peptide segments on the resin and their subsequent coupling to the above mentioned 9-aminoacridine diamine in solution was performed. This method led to a higher yield of crude peptide than the earlier one; however, contamination with its diastereoisomer was detected. Therefore, the yield of peptide after HPLC preparation was lower than that obtained using the convergent approach. Another route using the divergent approach was based on the synthesis of peptide segments forming *C*- and *N*-termini in solution. When Boc-Phe-Gln-OH was used in segment condensation, epimerization similar to that observed in the previous segment condensation occurred (Table 3). Therefore we finally decided in favor of coupling of Boc-Phe-Gln-Gly-OH and H-Ile$_2$-$NHCH_2CH_2NH$-Acr segments to avoid this epimerization and to obtain the H-Phe-Gln-Gly-Ile$_2$-$NHCH_2CH_2NH$-Acr in high yield.

Interaction of H-Phe-Gln-Gly-Ile$_2$-NH-CH_2-CH_2-NH-Acr conjugates with DNA was determined in a partition experiment between two polymers containing aqueous phases. According to Albertsson [27], dextran 500 000 and PEG 6000 were employed as water separators. The bottom phase contains higher amount of dextran and has higher affinity to DNA than the top one. The top phase is almost DNA-free and thus it serves as a reporter of free ligand concentration in the bottom one [28,29]. The binding constants are then evaluated from the following relations. An equilibrium between a binding site S_i of polynucleotide and ligand L given by Eqn (1) is characterized by thermodynamic stability constant K_i (Eqn (2)) where activities are replaced by concentrations. Equation (3) and total site concentration by Eqn (5) can express free ligand concentration.

$$S_i + L \rightleftharpoons S_iL \tag{1}$$

$$K_i = \frac{[S_iL]}{[S_i][L]} \tag{2}$$

$$[L] = C_L - \Sigma[S_iL] \tag{3}$$

$$C_{Si} = [S_i] + [S_iL] \tag{4}$$

$$C_{Si} = n_i C_N \tag{5}$$

where C_L stands for total ligand concentration, C_{Si} for total site concentration and C_N for the nucleotide concentration. The conjugate/DNA-binding ratio r is defined in Eqn (6). Two equations for r are given in Eqn (7) for noninteracting binding sites and Eqn (8) for interacting binding sites [30]. The cooperativity effect is covered by the Hill constant (α_H), which lies in the interval $1 \leq \alpha_H \leq n$. The nonlinear curve fitting of the dependency of r on [L] is the method for obtaining a binding constant (k_i), size of binding site (n_i) and the binding mode [31].

$$r = \frac{C_L - [L]}{C_N} \tag{6}$$

$$r = \sum \frac{n_i k_i [L]}{1 + k_i [L]} \tag{7}$$

$$r = \frac{n k^{\alpha H} [L]^{\alpha H}}{1 + k^{\alpha H} [L]^{\alpha H}} \tag{8}$$

The values of r and [L] may be easily obtained from equilibrium experiments after calibration of the partition coefficient as a function of total the ligand concentration, or the dependence of ligand fluorescence in the bottom phase (F_{bp}) on its fluorescence in the top phase (F_{tp}). Another constant that must be determined is the ratio of the top and bottom phase volumes (V_{tp}, V_{bp}). When the experiment is carried out with constant total concentration of the ligand and a variable concentration of the DNA, one observes a transition of the ligand from the top to bottom phase as decreasing fluorescence of the top phase. In the absence of DNA,

the total fluorescence of the system (F_{tot}) is given by Eqn (9).

$$F_{tot} = F_{bpmax} + \frac{V_{tp}}{V_{bp}} F_{tpmax} \quad (9)$$

In spite of the fact that the fluorescence of the bottom phase cannot be obtained directly in the presence of DNA, the value is obtained from correlation between the fluorescence in both the phases measured in the absence of DNA [($F_{bp} = f\ (F_{tp})$]. Now, we establish a virtual fluorescence of bound acridine conjugates (F_c) by Eqn (10).

$$F_c = F_{bpmax} - f\left(F_{tp}\right) + \frac{V_{tp}}{V_{bp}}\left(F_{tpmax} - F_{tp}\right) \quad (10)$$

Under these assumptions, we may write Eqns (11) and (12) for L and r, respectively, with only a single variable that is the virtual fluorescence of the complex.

$$[L] = \frac{C_L\,(F_{tot} - F_C)}{F_{tot}} \quad (11)$$

$$r = \frac{C_L F_c}{C_N F_{tot}} \quad (12)$$

Curve fitting applied to partitioning experiments gives the binding constant (Figures 3–5). Three models for DNA binding were evaluated. According to correlation coefficients, it seems that the best fitting is gained by the Hill model; however, the easiest fitting by a two parameter equation gives the result where Scatchard plots are used, which may be compared with the literature. Moreover, this correlation is only slightly poorer than that of Hill.

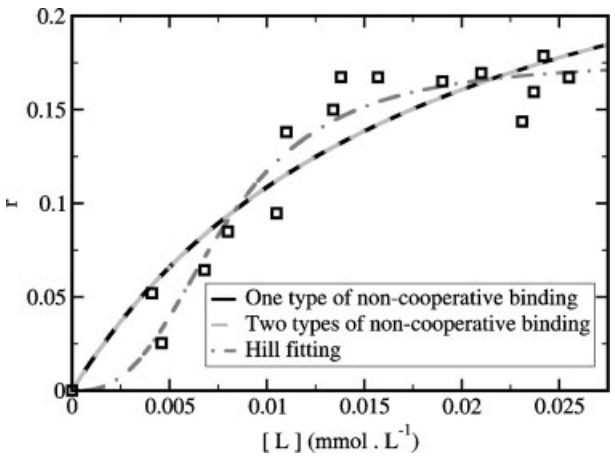

Figure 3 Titration of H-Phe-Gln-Gly-Ile$_2$-NHCH$_2$CH$_2$NH-Acr with CT-DNA in aqueous two-phase system. The data were evaluated by nonlinear fitting. Parameters of the Hill fitting (Eqn (8)): $n = 0.176364$; $k = 127.72$ l.mmol^{-1}; $\alpha_H = 2.77807$; correlation coefficient 0.968347. According to Eqn (7) with one kind of independent binding constant: $n_1 = 0.308484$; $k = 54.3221$ l mmol^{-1}; correlation coefficient 0.939858 and with two different kinds of independent binding constants: $n_1 = 1.80818.10^{-9}$; $k_1 = 1.10^{10}$ l mmol^{-1}; $n_2 = 0.308497$; $k_2 = 54.3177$ l mmol^{-1}; correlation coefficient 0.939857.

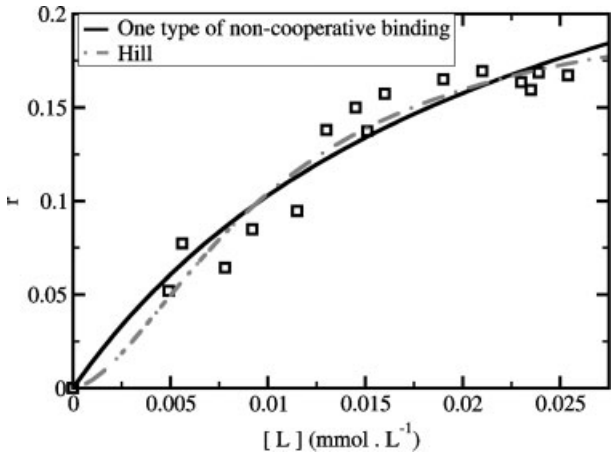

Figure 4 Titration of H-Phe-Gln-Gly-Ile$_2$ – εAhx-NHCH$_2$CH$_2$NH-Acr with CT-DNA in aqueous two-phase system. The data were evaluated by nonlinear fitting. Parameters of the Hill fitting (Eqn (8)): $n = 0.211323$; $k = 98.2419$ l mmol^{-1}; $\alpha_H = 1.66268$; correlation coefficient 0.973338. According to Eqn (7) with one kind of independent binding constant: $n = 0.336672$; $k = 43.9939$ l.mmol^{-1}; correlation coefficient 0.968508.

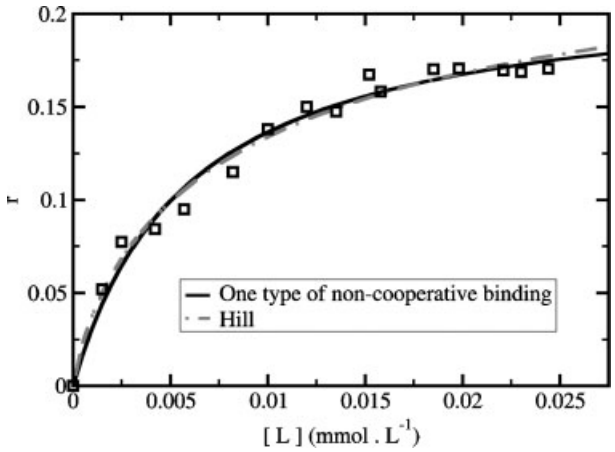

Figure 5 Equilibrium analysis of H-Gly-NHCH$_2$CH$_2$NH-Acr and CT-DNA in aqueous two-phase system. The data were evaluated by nonlinear fitting. Parameters of the Hill fitting (Eqn (8)): $n = 0.258014$; $k = 110.067$ l mmol^{-1}; $\alpha_H = 0.792055$; correlation coefficient 0.991631. According to Eqn (7) with one kind of independent binding constant: $n = 0.216667$; $k = 169.84$ l mmol^{-1}; correlation coefficient 0.990609.

Thus the combination of two strong DNA-binders – H-Phe-Gln-Gly-Ile$_2$-OH and 9-aminoacridine – in one molecule has led to the conjugate exhibiting a decreased binding constant with CT-DNA (Table 4). The same decrease in the binding activity was obtained on an extension of the binder length using 6-aminohexanoic acid. The influence of the prepared compounds on thermal stability of targeted oligonucleotide (Figure 1) is

small, if any (Table 5). Observation of no synergistic and/or binding effect indicated that in some suitable linkages of peptides with 9-aminoacridine the weaker DNA-binders can be obtained and thus the proposed concept of 'minimal intercalators' could be fulfilled, potentially enabling a sufficient extravascular distribution. If one molecule contains two different DNA-binding species and the final DNA-binding constant is lower than that of the model peptide, 9-aminoacridine conjugate H-Gly-NH-CH_2-CH_2-NH-Acr, then we can assume that competitive binding occurs. Therefore, it seems possible that the DNA-binding moieties of the prepared compound could play a competitive role and cleave the formed complex. Thus the newly prepared structures might be potentially useful as the mild tuners of DNA interaction with strong bis-acridine binders.

CONCLUSIONS

We report on the preparation and binding characteristics of synthetic peptide conjugates containing 9-aminoacridine. These conjugates appear to be weak DNA-binders. The attachment of two DNA-binding moieties led to molecules with weaker binding affinity than common DNA intercalators. Our explanation for the effect is in the hypothesis that the individual binding parts of the molecule play a self-competitive role in the DNA interaction. Thus, the synthesis of the new DNA-binders with the minimal binding activity fulfills a requirement for the mandatory binding mode combined with the lowest possible level of binding, which would not limit diffusion of the drug in the body driven by the free drug concentration and could maintain necessary extravascular distribution, as well.

Acknowledgements

This research was carried out under the research project Z40550506 and was supported by the Grant Agency of the Czech Republic (grant No. 203/02/1379). We thank Dr. I. Rosenberg from the Institute of Organic chemistry and Biochemistry for performing the experiments on synthesis and melting of the oligonucleotide.

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