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DNA Sequence-Specific Ligands: XI.* The Synthesis and Binding to DNA of bis-Netropsins with the *C*-Ends of Their Netropsin Fragments Tethered by Tetra- or Pentamethylene Linkers

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Abstract—Bis-Netropsins with the *C*-ends of their netropsin fragments tethered via tetra- or pentamethylene linkers and with Gly or *L*-Lys-Gly residues on their *N*-ends were synthesized. The footprinting technique was used to study the specificity of bis-netropsin binding to the specially constructed DNA fragments containing various clusters of A · T pairs. It was found that the linker length affects the binding of bis-netropsins, with the tetramethylene linker providing better protection than the pentamethylene linker. It was shown that the newly synthesized bis-netropsins bind tighter to the 5'-$\mathbf{A_4T_4}$-3' sequence, whereas the bis-netropsin with a linker between the netropsin *N*-ends binds better to 5'-$\mathbf{T_4A_4}$-3' sequences.

Key words: *bis-netropsins, chemical synthesis, distamycin A, DNA sequence-specific ligands, footprinting, netropsin*

INTRODUCTION

Natural antibiotics netropsin and distamycin A form tight noncovalent complexes with A · T-enriched regions of double-helical DNA [2–10].[3] A structure of a new class of compounds that should preferably recognize certain clusters of DNA A · T pairs was suggested on the basis of a molecular model of netropsin– and distamycin A–DNA complexes, according to which antibiotic molecules are disposed in the complexes along the DNA minor groove [4]. A general strategy for constructing the compounds of this type is the introduction of various linkers between one or several pyrrolecarboxamide groups, which allows a definite orientation and fixing of these fragments in the DNA complex.

Bis-Netropsins constituted of two molecules of a netropsin analogue whose *N*-ends were tethered by the residues of aliphatic dicarboxylic acids with various numbers of methylene groups were the first compounds of this class, synthesized in 1982 [11]. The study of physicochemical [6, 12] and biochemical [6, 13–16] properties of their complexes with DNA showed that the netropsin fragments are specifically bound to two clusters of A · T pairs. Subsequently, the sequence-specific compounds on the basis of various combinations of pyrrole- and imidazolecarboxamide building blocks were successfully developed in other laboratories [7, 17–20].

We report herein the synthesis and the specificity of DNA binding of bis-netropsins in which the *C*-ends of netropsin fragments are linked by the residues of α,ω-diaminoalkanes of various lengths and contain Gly or *L*-Lys-Gly residues on their *N*-ends.

RESULTS AND DISCUSSION

Chemical formulas of netropsin, distamycin A, and bis-netropsins we synthesized [(**I**), (**Ia**), (**II**), and (**IIa**)] are shown in Fig. 1. Bis-Netropsins contain two netropsin fragments that are built of two *N*-propylpyrrolecarboxamide blocks linked by five, (**I**) and (**II**), or four (**Ia**) and (**IIa**), methylene groups. Each of the di-*N*-propylpyrrolecarboxamide blocks differs from the netropsin molecule not only in the structure of its *C*-terminal residue and the substitution of *n*-propyl for methyl groups at the pyrrole nitrogen atoms, but also in the substitution of Gly [in (**I**) and (**Ia**)] or *L*-Lys-Gly [in (**II**) and (**IIa**)] residues for the guanidylacetic acid residue. According to the X-ray analysis of oligonucleotide complexes with netropsin [21] or distamycin A [22], these structural changes should not affect the specificity of these compounds toward the DNA A · T pairs, since this specificity results from the formation of hydrogen

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[3]Abbreviations: Apc, 1-propyl-4-aminopyrrole-2-carbonyl residue; CDI, *N*,*N*'-carbonyldiimidazole; HOBt, 1-hydroxybenzotriazole; Im, imidazole residue; Npc, 1-propyl-4-nitropyrrole-2-carbonyl residue. All the nucleotide sequences are given in 5' ⟶ 3' direction.

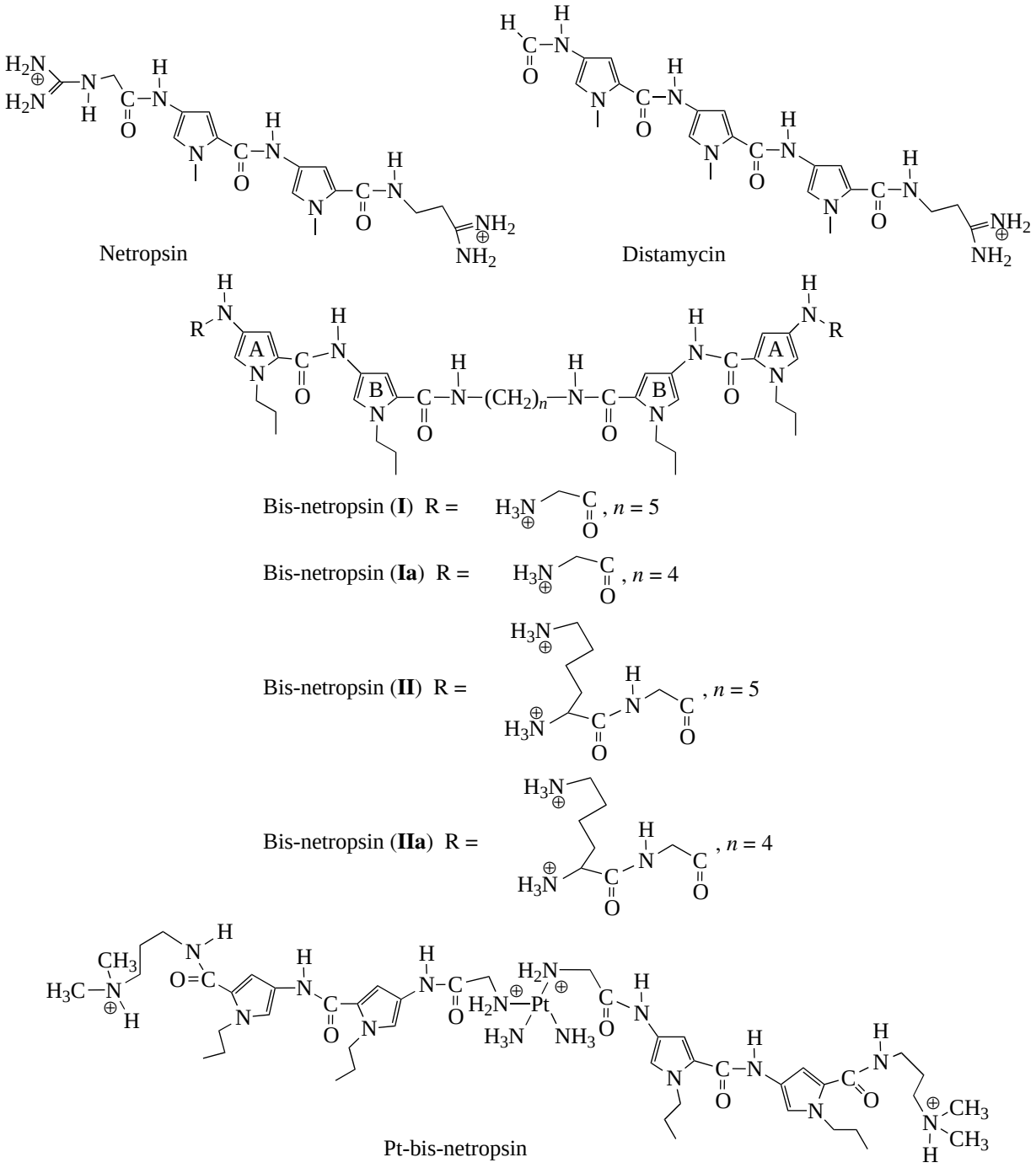
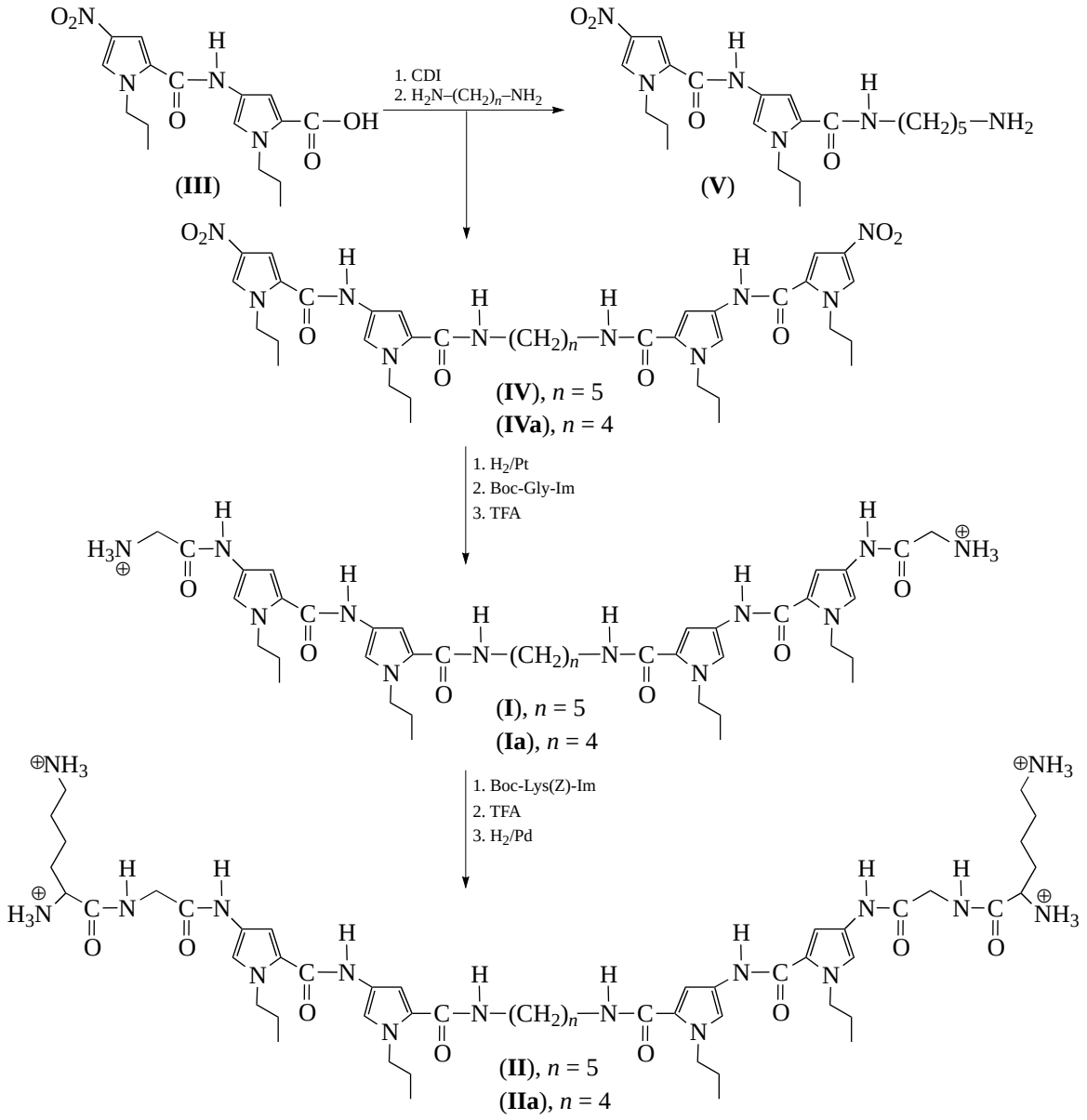


Fig. 1. Chemical structures of netropsin, distamycin, and bis-netropsins

bonds between nitrogen atoms of the ligand amide groups and acceptor groups of base pairs in the DNA minor groove. In addition, the yields of *N*-propyl derivatives of 4-nitro- and 4-aminopyrrole-2-carboxylic acids are higher in comparison with the yields of the corresponding *N*-methyl derivatives [3], and the replacement of the terminal guanidine group by amino group enables its further modifications when obtaining more complex DNA-specific ligands.

Synthesis of bis-Netropsins

Bis-Netropsins (**I**) and (**II**) with pentamethylene linker were synthesized according to Scheme 1; a sim-

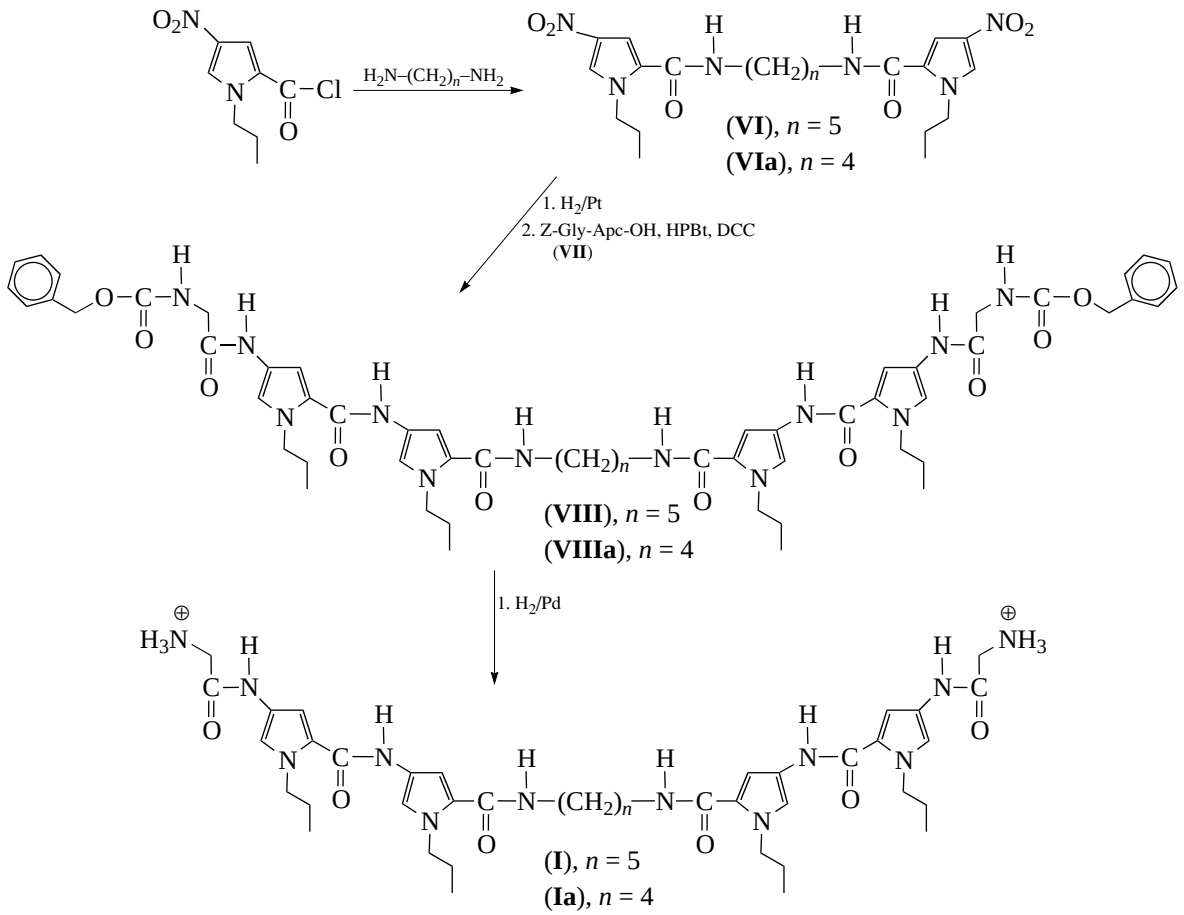


Scheme 1.

ilar scheme was used for bis-netropsins with the tetramethylene linker. Hereinafter, the homologous compounds with 1,4-tetramethylendiamine (putrescine) linker in place of a 1,5-pentamethylendiamine (cadaverine) linker are marked with index **a**. Imidazolide of (**III**), synthesis of which was previously described [23], was coupled with cadaverine in DMF. The yields of dinitroderivative (**IV**) and its lower homologue (**IVa**) were 59 and 46%, respectively. When synthesizing (**IV**), we also isolated a monoacylcadaverine derivative (**V**) in 12% yield. After reduction of nitro groups in (**IV**) and (**IVa**) by hydrogenation in the presence of Adams catalyst, the resulting diamino derivatives were coupled *in situ* with imidazolide of Boc-glycine. The protective groups were removed to give (**I**) and (**Ia**) in 32 and 24% yields, respectively.

The resulting compounds were poorly soluble in water under neutral pH. The introduction of two Lys residues at Gly amino groups using *N*,*N*'-carbonyldiimidazole substantially improved the solubility of bis-netropsins (**II**) and (**IIa**).

Bis-Netropsins (**I**) and (**Ia**) were also synthesized according to Scheme 2. The coupling of 1-propyl-4-nitropyrrole-2-carbonyl chloride with cadaverine or putrescine and the subsequent reduction of intermediate (**VI**) and (**VIa**) over the Adams catalyst led to the corresponding diaminoderivatives, which were coupled *in situ* with Z-Gly-Apc-OH (**VII**) using the carbodiim-



Scheme 2.

ide method. Bis-Netropsins (**I**) and (**Ia**) were prepared by catalytic hydrogenation of intermediate (**VIII**) and (**VIIIa**), respectively, over a palladium catalyst. Overall yields of bis-netropsin (**I**) according to Schemes 1 and 2 were 19 and 28%, respectively; and those of bis-netropsin (**Ia**), 11 and 24%, respectively.

The Specificity of bis-Netropsin Binding to DNA

In order to determine the DNA sequences to which bis-netropsins are most affine, the sequences with the highest affinity to the netropsin fragment that consists of two pyrrolecarboxamide blocks should preliminarily be found. According to X-ray analysis of netropsin and distamine complexes with various double-helical oligodeoxynucleotides, the amide groups of the antibiotics can form a bifurcated (three-centered) hydrogen bond with the neighboring N3 atoms of adenine and/or O_2 atoms of thymine in adjacent A · T pairs of complementary DNA strands [21, 22]. The NMR data [24] and footprinting analysis using DNase I [14, 25–28] and hydroxyl radicals [29] suggest that netropsin binds to the site composed of four A · T pairs in the DNA minor groove. The constants of netropsin binding to such sites depend on the mutual disposal of adenine and thymine residues in complementary DNA strands [26]. The tightest binding was observed for the sites containing thymine residues only in one of the strands and for the sites containing the AT sequence. The presence of the TA sequence in the site weakens the binding [26]. Conformational calculations [30], X-ray data [31], and the results of the DNA cleavage with hydroxyl radicals [32] suggest that the minor grooves of the binding sites containing the TT sequences or the AT sequence are narrowed and their size corresponds to the thickness of netropsin molecule. When being inserted in this groove, netropsin forms numerous van der Waals contacts, which give rise to a tight complex [24]. These considerations are also valid for the case of distamycin binding to DNA; the only exception is that it recognizes five rather than four A · T pairs.

The binding sites containing pyrimidine–purine TA and/or GC sequences have the widened minor groove because of small overlapping of neighboring base pairs. For distamycin and some of its analogues, the side-by-side parallel insertion of two antibiotic molecules into the widened minor groove was found using NMR spectroscopy [33]. The monomeric binding of netropsin and

distamycin to the sequences containing G · C base pairs is energetically unfavorable because of steric hindrances resulting from the guanine 2-amino group situated in the minor groove [4, 21], whereas the dimeric binding is possible in the case of sites with widened minor grooves [34].

The ligand molecule can be incorporated into the minor groove in two orientations in the process of monomeric binding to nonpalindromic DNA sequences, for example, to the TTTT sequence [24]. The introduction into the terminal regions of netropsin or distamycin molecules of a group capable of cleaving the DNA sugar–phosphate backbone allows the determination of both the binding specificity and the orientation of the molecule in the minor groove [35–37]. In the case of the TTTT sequence, netropsin analogues are predominantly oriented along the groove in such a manner that their amidine moiety (the *C*-end) is situated at the 5'-end of this sequence. Therefore, the bis-netropsins, in which two netropsin fragments are in different orientations, should have the highest affinity to different sites. In addition, as follows from the behavior of the duplexes containing octanucleotides with reversed polarities (A_4T_4 and T_4A_4) using the gel mobility shift assay in PAG [38] and molecular modeling [30, 39], the polarity of a nucleotide sequence can be an important parameter that determine the DNA structure and curvature, which can affect the binding of extended ligands along the DNA minor groove.

In addition to the contribution of each of the two netropsin fragments, the nature of linker also dramatically affects the bis-netropsin affinity to various DNA sequences. Several structures of DNA–bis-netropsin complexes are possible in the minor groove:

(1) Both netropsin fragments form specific complexes with DNA. To realize this possibility, DNA should contain two closely situated recognition sites, and linker should not prevent them from mutual binding. An excessively long linker can lead to the possibility of binding of the second half of bis-netropsin to several closely situated sites. To reduce the number of possible variants, we chose comparatively short linkers, although this increased the probability of preventing the specific binding of one block by the binding of another one.

(2) If there is only one binding site or the linker hinders binding of the second half of bis-netropsin, only one of the netropsin fragments would tightly bound by DNA, whereas another one would either be expelled out of the groove or be inside it, but would not form specific contacts.

(3) For bis-netropsins, the side-by-side insertion in the minor groove of two netropsin fragments and formation of pins are possible.

(4) The binding and simultaneous side-by-side incorporation of netropsin fragments from different bis-netropsin molecules in the same binding site is also possible.

Using statistical analysis of a large number of binding sites, we previously found that Pt-bis-netropsin in which two netropsin fragments were linked through their *N* termini via *cis*-diammineplatinum(II) linker (Fig. 1) forms the tightest complex with the T_4A_4 sequence [40]. In bis-netropsin molecules (**II**) and (**IIa**), the netropsin fragments linked by their *C*-ends are oriented in opposite directions. Therefore, one can expect that these bis-netropsins would form tighter complexes with the A_4T_4 sequence.

We constructed plasmids bearing several potential binding sites in the polylinker part in order to study the binding of various bis-netropsins by DNA [28]. Each site contains two netropsin binding sites that include various sequences formed by four- or five A · T base pairs either closely disposed or separated by one, two, or three G · C pairs. These plasmid regions included the sequences containing four A · T pairs in various combinations and surrounded with the G · C-enriched clusters. The sequences of two constructed DNA fragments **A** and **B** are shown in Fig. 2.

*The Specificity of Binding of bis-Netropsins (**II**) and (**IIa**) to the DNA Fragment **A***

The patterns of cleavage of the upper strand of radioactively labeled DNA fragment **A** by hydroxyl radicals and by the DNase I in the presence of bis-netropsins (**II**) and (**IIa**) at various concentrations are shown in Figs. 3 and 4, respectively. One can see in the patterns that all of the four above discussed variants of the structures of bis-netropsin–DNA complexes appear to be realized in the presence of bis-netropsins (**II**) and (**IIa**). We observed that, at ligand concentrations of 8–10 μM, which corresponds to approximately one ligand molecule per four DNA base pairs, the whole fragment **A** and even the GC sequences in it were strongly protected from the cleavage. Under these saturation conditions, the formation of various types of dimeric or multimeric [41] poorly specific complexes is possible.

If the bis-netropsin concentration did not exceed 4–5 μM, they protected from cleavage only the sites containing no less than four successive A · T pairs. Bis-Netropsin (**IIa**) containing the tetramethylene linker was a more potent protector than bis-netropsin (**II**) with pentamethylene linker (Fig. 4).

These compounds are most tightly bound to the sequence A_5T_5 [site (4)]: at the concentrations of 1.2 and 0.6 μM, respectively, bis-netropsins (**II**) and (**IIa**) protect from cleavage about 7 bp. Bis-Netropsin (**IIa**) at the concentration of 2.5 μM protects from cleavage only 4-bp region situated at 3' end in sequences T_5A_5 [site (6)] and T_5CA_5 [site (2)]. As in the case of the TTAT sequence [site (3)], this suggests the formation of a hairpin-like structure [42]. An indirect proof of this fact is the intensification of the effect of hydroxyl radicals of the neighboring sites, which implies the broadening of the minor groove. This intensification was not

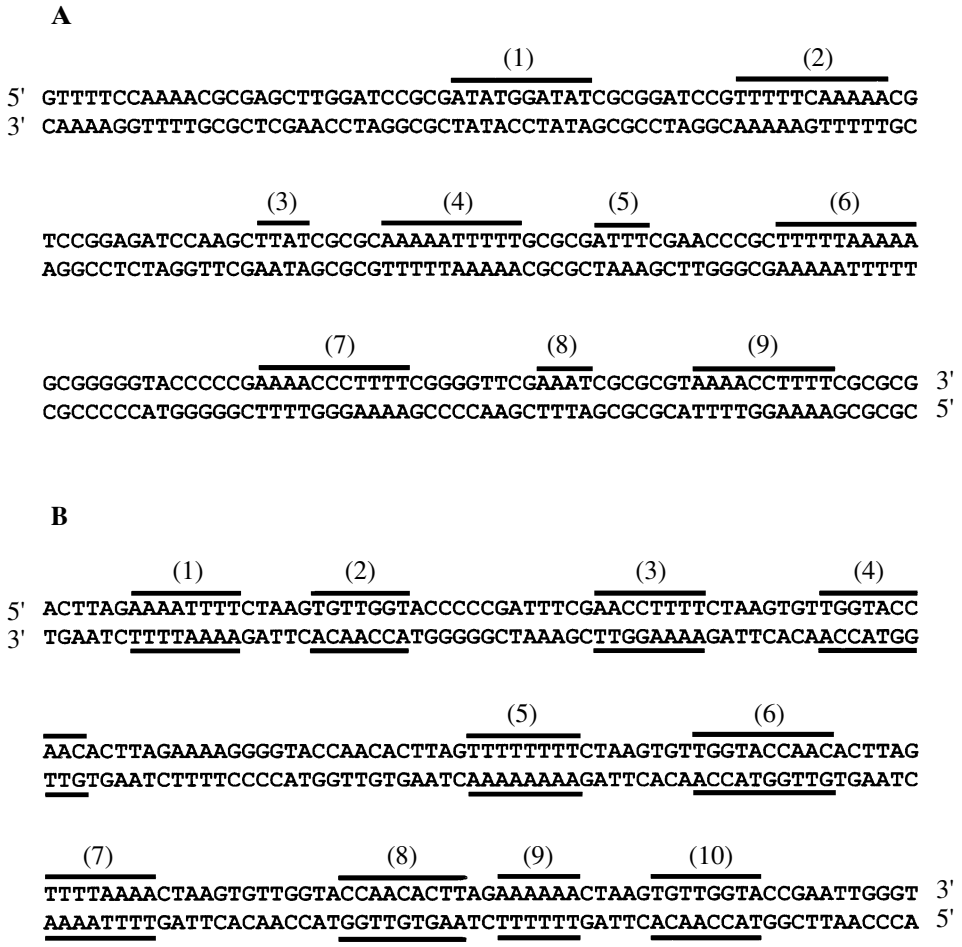


Fig. 2. Nucleotide sequences of DNA restriction fragments **A** and **B**. The sites designated in Figs. 3–7 are marked and numbered.

observed in the case of the AAAT sequence [site (8)]; in this case, only one of the netropsin fragments is probably involved in the binding. In the ATATGGATAT sequence [site (1)], (**IIa**) protects about 10 bp, with a weak cleavage being observed in the center of the sequence. In this case, two bis-netropsin molecules appear to be bound in the form of hairpins [43].

As a whole, these data suggest that bis-netropsins (**II**) and (**IIa**) are more affine to the A_5T_5 than to the T_5A_5 sequence. Nevertheless, this difference is much weaker pronounced than in the case of Pt-bis-netropsin containing netropsin fragments in opposite orientations and specifically affine to the sequence T_5A_5 [40]. This may be due to inability of the linkers under study to ensure the optimal transition for "reading" two AT clusters with two netropsin fragments in opposite orientations. On the other hand, Pt-bis-netropsin not only exhibits the optimal orientation of netropsin fragments, but also has a bulky *cis*-diammineplatinum group that well incorporated into the TA-determined widening of the minor groove and enables each of the netropsin blocks to form tight specific contacts.

*The Binding Specificity for Netropsins (**II**) and (**IIa**) to the DNA Fragment **B***

Since the analysis of the sites protected in fragment **A** revealed that bis-netropsins (**II**) and (**IIa**) are bound by the sequences containing alternating A · T and G · C base pairs, we analyzed similarly the fragment **B** containing many such sequences. Figures 5 and 6 present the patterns of cleavage with DNase I of the upper and lower strands, respectively, of radioactively labeled fragment **B** at various concentrations of bis-netropsins (**II**) and (**IIa**). The cleavage patterns of the lower strand of fragment **B** with hydroxyl radicals are shown in Fig. 7.

One can see in Figs. 5–7 that bis-netropsin (**IIa**) with tetramethylene linker, unlike bis-netropsin (**II**) with pentamethylene linker, is bound, (e.g. Fig. 5), by TGGTACCAC [site (4)], AACCTTTT [site (3)],

TGTTGGT [site (2)], and some other similar sequences at the concentration of 2.5–5.0 μM. The binding by these sequences is most probably due to the formation of hairpin-like structures. Thus, in fragment **B**, bis-netropsin (**IIa**) exhibits a more strong protection from cleavage than bis-netropsin (**II**) whose linker is only one methylene unit longer.

EXPERIMENTAL

Solutions of substances in organic solvents were dried over Na_2SO_4. Solvents were removed on a rotary evaporator at 20°C in the vacuum of a water-jet or oil pumps. Compounds were dried in a vacuum over P_2O_5 and NaOH. Melting points were determined on a Boethius device (Germany) and were not corrected. Hydrogenation was carried out over the Adams catalyst [44] at atmospheric pressure and 25°C until a vigorous hydrogen absorption was ceased. Purity of the resulting compounds was monitored by TLC on Silufol UV-254 precoated plates (Czech Republic) in the following systems: 4 : 1 : 1 *n*-BuOH–AcOH–water (A); 7 : 1 : 3 Pr*i*OH–conc. ammonia–water (B); 3 : 7 1 M $AcONH_4$ (pH 7.6)–EtOH (C); and 95 : 5 $CHCl_3$–absolute EtOH (D). The spots of substances were detected on chromatograms according to the UV-light absorption at 254 nm or with ninhydrin. If not stated otherwise, the column chromatography was carried out on a Sephadex LH-20 (Pharmacia, Sweden) column (3 × 150 cm) eluted with methanol at 4°C and a rate of 15 ml/h; 15-ml fractions were collected. The elution was monitored using a UV flowing densitometer Uvicord II-8300 (Sweden). Final purification of (**I**), (**Ia**), (**II**), and (**IIa**) and analysis of their homogeneity was carried out by the reversed-phase HPLC on a silica gel C-18 (6 μm) column (250 × 15 mm) (SKB IOKh AN SSSR) under elution by a linear gradient of acetonitrile in 0.1% TFA (0–80% for 45 min; flow rate 4.8 ml/min). HPLC was performed on a Gilson chromatograph (France) comprising model 302 and 303 pumps, a model 803C manometer, a 811 mixer, a Data Master-621 control unit, and a model 117 UV detector. The light absorption of eluates was determined at 240 and 300 nm.

^{1}H NMR spectra were registered on a Varian XL-100 spectrometer (United States) with a working frequency of 100 MHz at 33°C in DMSO-d_6 with Me_4Si as an internal standard (δ 0.00). Proton chemical shifts (δ) are given in ppm. The symbols of pyrrole cycles (A, B) are depicted in Fig. 1.

Mass spectra were registered on an MS-50TC spectrometer (Kratos, UK) using the fast atom bombardment with xenon at 6–8 keV and glycerol as a matrix.

DNA fragments were prepared by digestion of a modified plasmid pGEM7(f+) (Promega) containing an insert of synthetic oligonucleotides within the polylinker with restriction endonucleases *Xba*I and *Nsi*I (Promega) (fragment **A**) [28] or *Eco*RI and *Hin*dIII (Promega) (fragment **B**) [16]. The radioactive label was

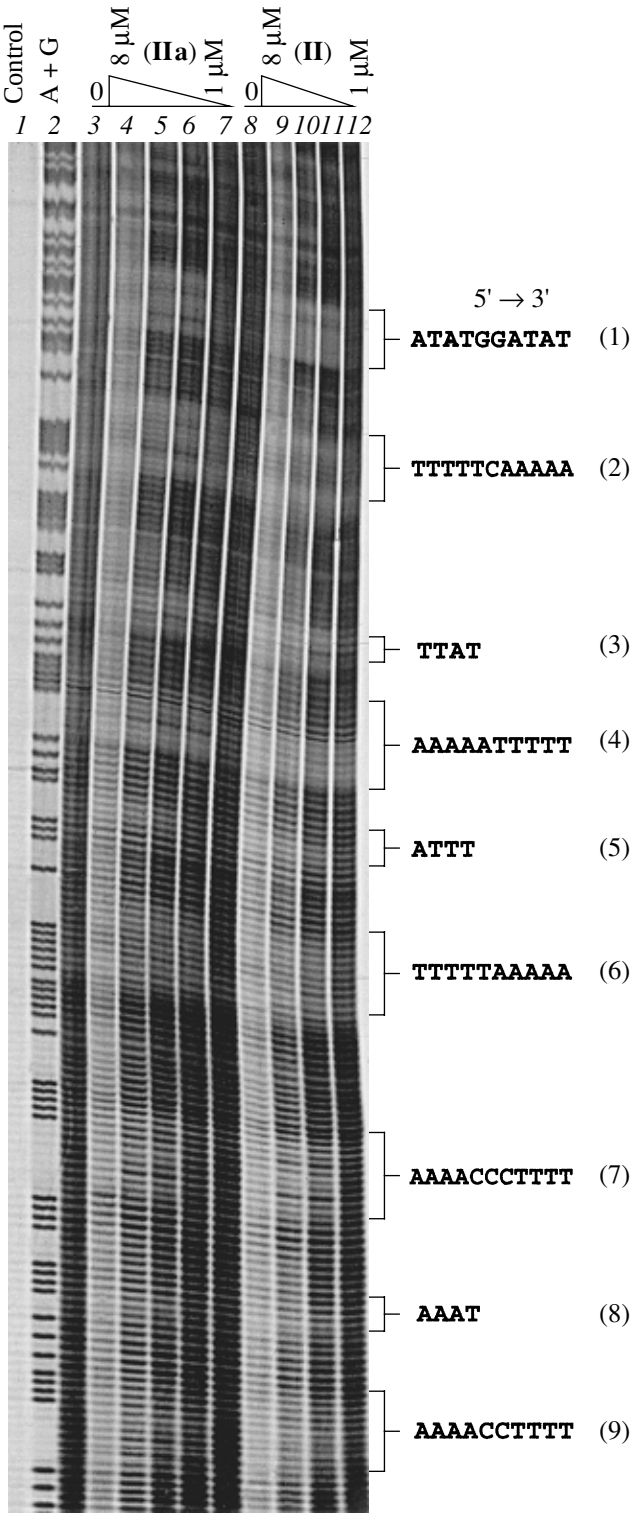


Fig. 3. The patterns of cleavage with hydroxyl radicals of the upper DNA fragment **A**: *1*, initial (untreated) fragment; *2*, A + G, chemical cleavage at purine residues; *3* and *8*, the cleavage of the free fragment; *4–7* and *9–12*, the fragment cleavage in the presence of 8, 4, 2, and 1 μM bis-netropsins (**IIa**) and (**II**), respectively.

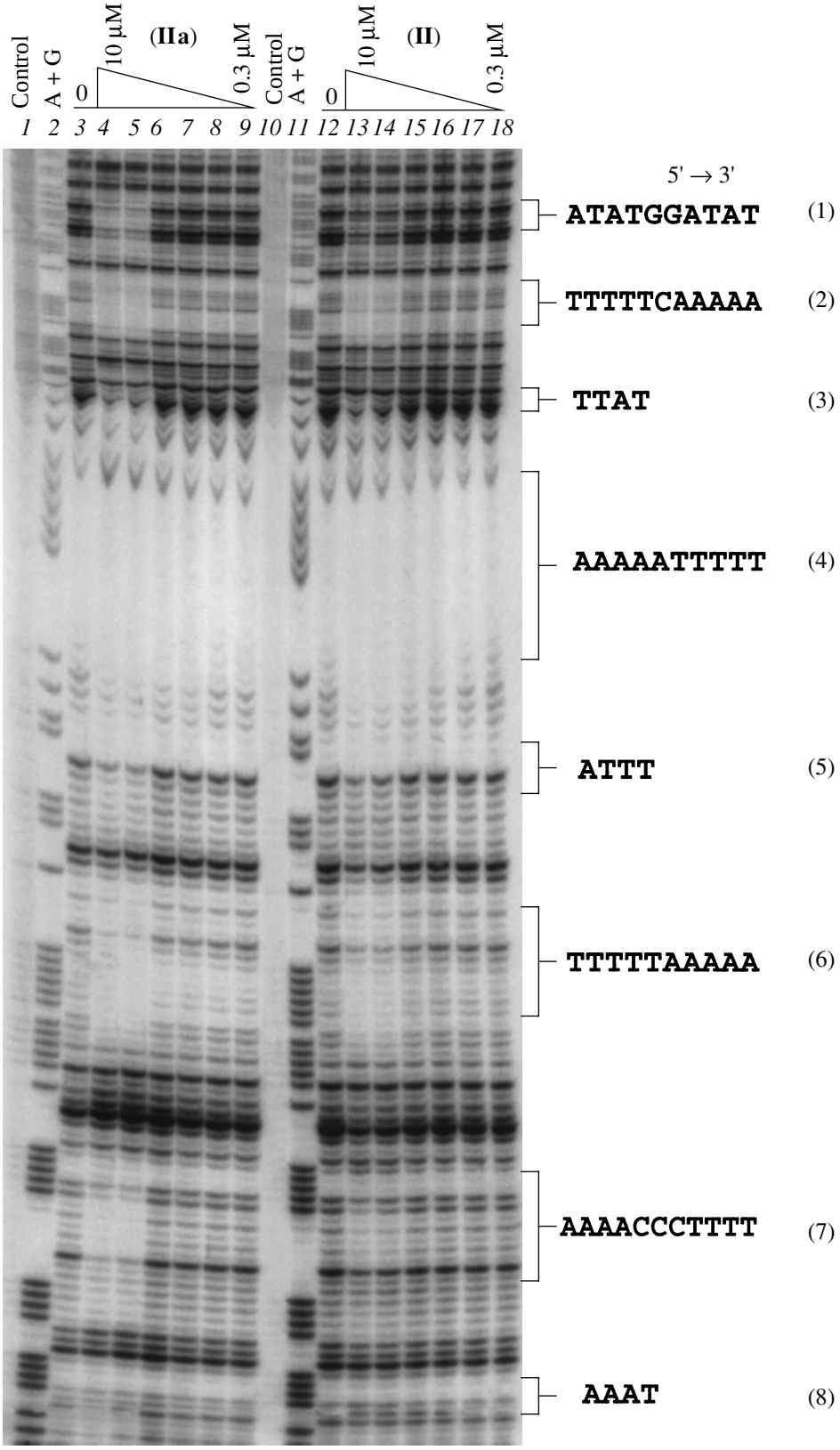


Fig. 4. The patterns of cleavage with DNase I of the upper DNA fragment **A**: *1* and *10*, initial (untreated) fragment; *2* and *11*, chemical cleavage at purine residues; *3* and *12*, cleavage of the free fragment; *4–9* and *13–18*, the fragment cleavage in the presence of 10, 5, 2.5, 1.25, 0.62, and 0.31 μM bis-netropsins (**IIa**) and (**II**), respectively.

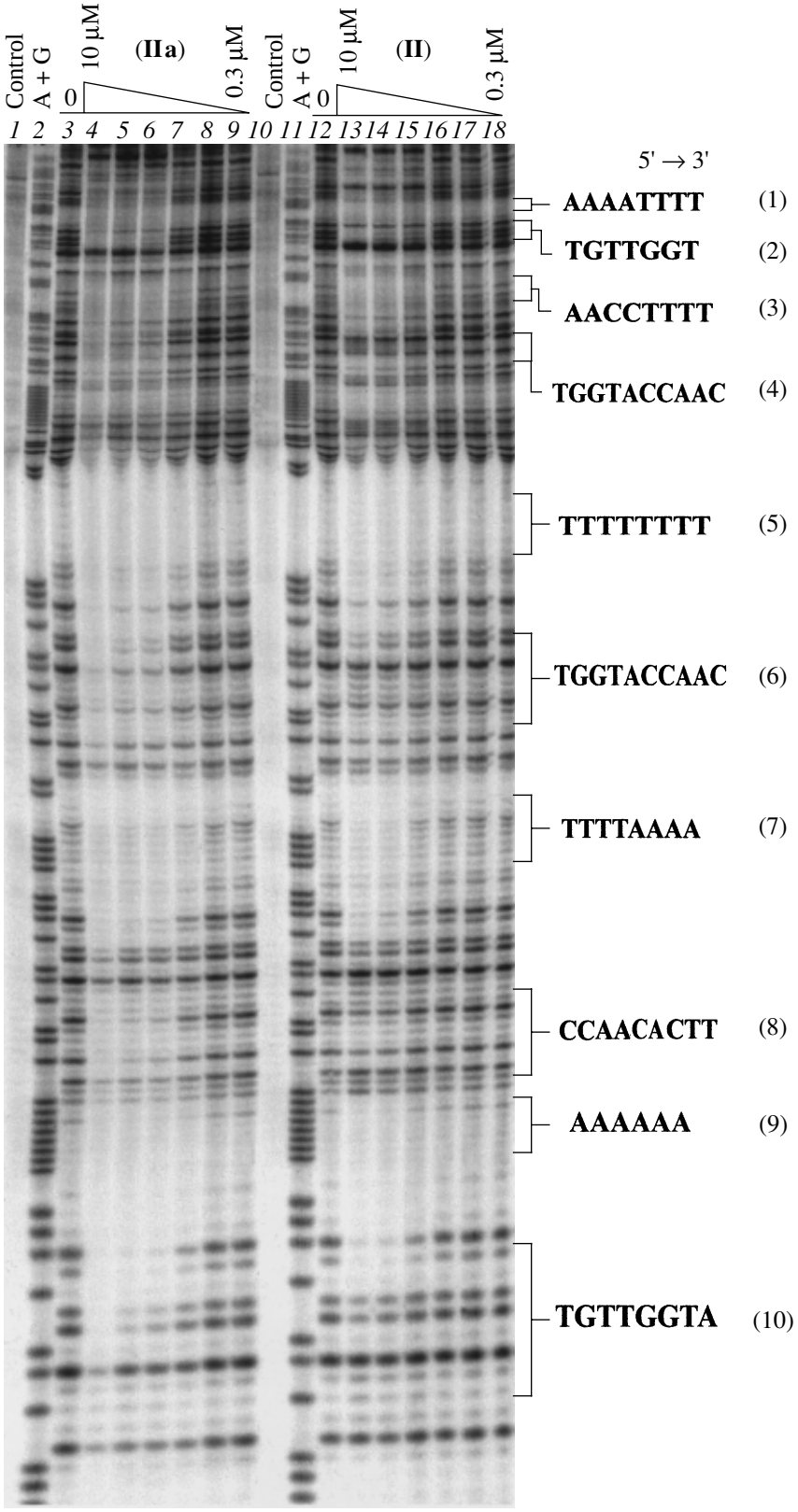


Fig. 5. The patterns of cleavage with DNase I of the upper DNA fragment **B**: *1* and *10*, initial (untreated) fragment; *2* and *11*, chemical cleavage at purine residues; *3* and *12*, cleavage of the free fragment; *4–9* and *13–18*, the fragment cleavage in the presence of 10, 5, 2.5, 1.25, 0.62, and 0.31 μM bis-netropsins (**IIa**) and (**II**), respectively.

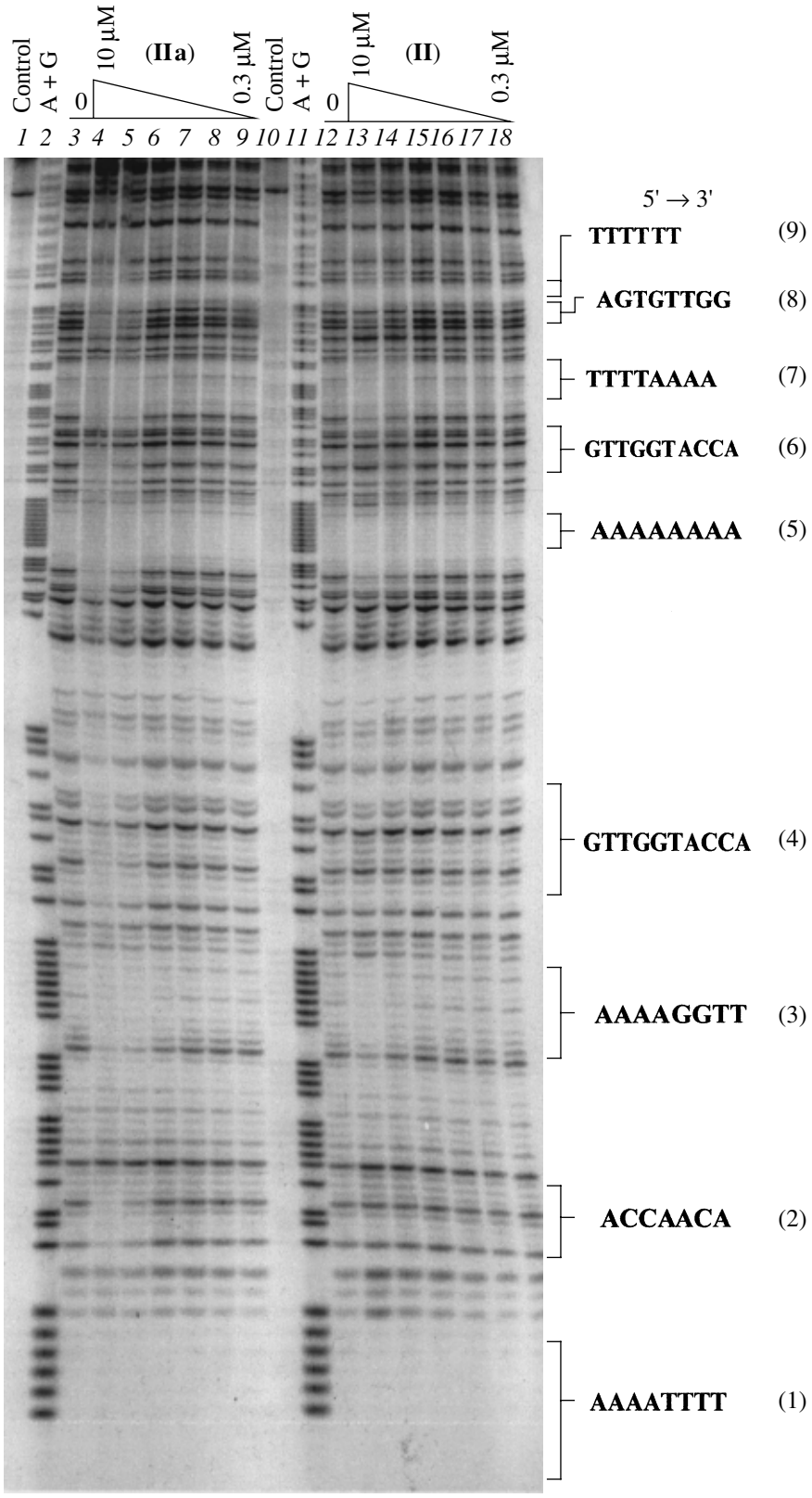


Fig. 6. The patterns of cleavage with DNase I of the lower DNA fragment **B**: *1* and *10*, initial (untreated) fragment; *2* and *11*, chemical cleavage at purine residues; *3* and *12*, cleavage of the free fragment; *4–9* and *13–18*, the fragment cleavage in the presence of 10, 5, 2.5, 1.25, 0.62, and 0.31 μM bis-netropsins (**IIa**) and (**II**), respectively.

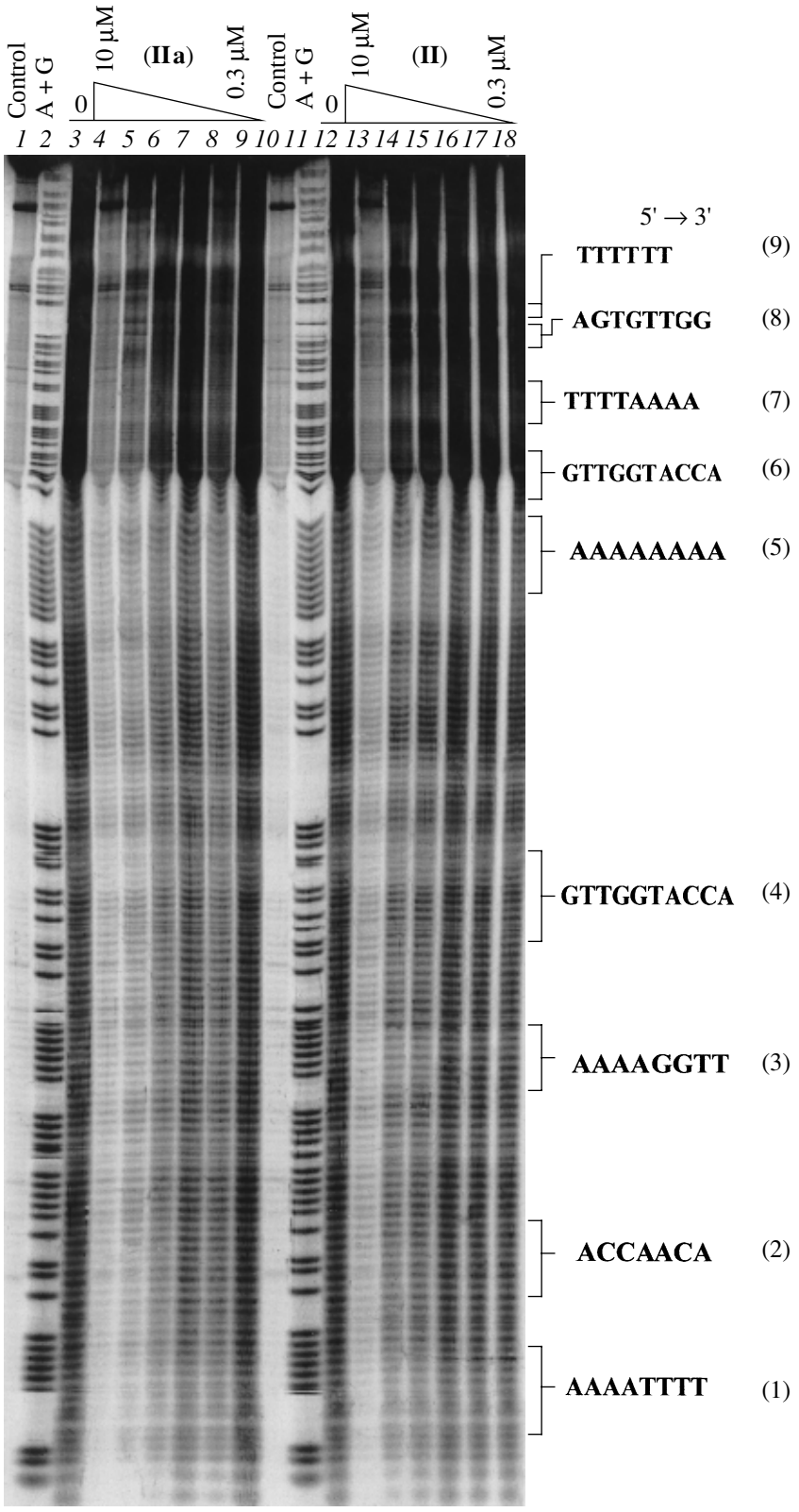


Fig. 7. The patterns of cleavage by hydroxyl radicals of the lower DNA fragment **B**: *1* and *10*, initial (untreated) fragment; *2* and *11*, chemical cleavage at purine residues; *3* and *12*, cleavage of the free fragment; *4–9* and *13–18*, the fragment cleavage in the presence of 10, 5, 2.5, 1.25, 0.62, and 0.31 μM bis-netropsins (**IIa**) and (**II**), respectively.

introduced into 3'-end of the fragment using [α-^{33}P]dCTP (fragment **A**) or [α-^{33}P]dATP (fragment **B**) (Isotop, St. Petersburg), unlabeled dNTP, and Klenow fragment of *Escherichia coli* DNA polymerase I, (Boehringer Mannheim, Germany) [45]. DNA fragments were isolated by PAGE in 5% PAG.

Footprinting by means of hydroxyl radicals [46]. To prepare the complex, a solution of the fragment (5 μl, about 10^4 Bq) and 6 μM (per 1 kbp) unlabeled DNA plasmid pGEM(f+) in 10 mM Tris-HCl (pH 7.5) was mixed with a ligand solution (5 μl) (concentrations are given in figure captions) in the same buffer, and the mixture was cooled to 0°C. A solution (5 μl) containing 1 mM EDTA, 0.5 mM Moore salt, 20 mM sodium ascorbate, and 10 mM Tris-HCl (pH 7.5) and 0.2% solution of H_2O_2 (5 μl) were simultaneously added, and the mixture was kept for 2 min at 0°C. The reaction was quenched by a solution (85 μl) containing 0.15 M NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 10 μg/ml tRNA. The mixture was extracted with phenol, the DNA was precipitated with ethanol, washed with 70% ethanol, dried, and dissolved in 95% formamide (1 μl) containing 15 mM EDTA (pH 8.0), 0.05% Bromphenol Blue, and 0.05% Xylene Cyanol FF. The mixture was heated for 1 min at 90°C, rapidly cooled, and loaded on a 8% denaturing PAG (40 cm long) with a gradient thickness of 0.15–0.45 mm [45, 47]. PAGE was performed for 70 min at 90 W (2.5 kV). Prior to superimposition, the gel was fixed in 10% acetic acid and dried on a glass plate preliminarily treated with γ-methacrylpropyloxysilane (LKB, Sweden).

Footprinting with DNase I. To prepare the complex, a solution of a fragment (5 μl, about 10^4 Bq) and a solution of 6 μM (per 1 kbp) unlabeled DNA of plasmid pGEM7(f+) (Promega) in 10 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl were mixed with a ligand solution (concentrations are given in figure captions) in the same buffer and cooled to 0°C. A solution (10 μl) of DNase I (1 μg/ml) (Sigma) in 10 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl and 5 mM $MnCl_2$ were added, and the mixture was kept for 2 min at 0°C. Further treatment was performed as described in the above procedure.

Npc→Apc→NH(CH$_2$)$_5$NH←Apc←Npc (IV). Anhydrous DMF (40 ml) was added to NpC-Apc-OH (**III**) (10.0 g, 28.7 mmol) [23] and CDI (28.7 mmol), and the mixture was stirred for 30 min at 20°C. A solution of cadaverine (14.4 mmol, 1.7 ml) in DMF (10 ml) was added in portions (2 ml/h) to the resulting imidazolide. The reaction mixture was kept for 70 h at 20°C and evaporated. The residue was dissolved in ethyl acetate (300 ml) and the solution was washed with 1 M HCl (3 × 100 ml), water, and saturated $NaHCO_3$ solution (3 × 100 ml); dried and evaporated. The residue was dissolved in methanol (100 ml) and kept overnight at –10°C. The precipitate was filtered and washed with methanol and ether to give (**IV**); yield 6.5 g (59%); mp 148–150°C, R_f 0.97 (A) and 0.19 (D); ^{1}H NMR: 10.29 (2 H, s, ^{A}CONH^B), 8.23 (2 H, d, H5^A), 8.03 (2 H, t, CONHCH$_2$), 7.60 (2 H, d, H3^A), 7.25 (2 H, d, H5^B), 6.87 (2 H, d, H3^B), 4.40 (4 H, t, N^{A}CH$_2$), 4.25 (4 H, t, N^{B}CH$_2$), 3.19 (4 H, m, NHCH$_2$CH$_2$), 1.70 (8 H, m, CH$_2$CH$_3$), 1.48 (6 H, m, CH$_2$), and 0.81 (12 H, m, CH$_3$); MS, *m/z*: 763 [*M* + H]$^+$; calc. for $C_{37}H_{50}N_{10}O_8$: 762.87.

To isolate the monoacyl derivative of cadaverine (**V**), the combined acidic extracts were neutralized with $NaHCO_3$, and extracted with ethyl acetate. The organic phase was dried and precipitated with a solution of 1 M hydrogen chloride in ethyl acetate. The resulting oil was separated, dissolved in methanol, and purified on a Sephadex LH-20 column to give (**V**) hydrochloride (1.6 g, 12%); mp 214–217°C; R_f 0.48 (A) and 0.52 (B); ^{1}H NMR: 10.36 (2 H, s, ^{A}CONH^B), 8.25 (1 H, d, H5^A), 8.09 (1 H, m, CONHCH$_2$), 7.68 (1 H, d, H3^A), 7.28 (1 H, d, H5^B), 6.93 (1 H, d, H3^B), 4.42 (2 H, t, N^{A}CH$_2$), 4.26 (2 H, t, N^{B}CH$_2$), 3.18 (2 H, m, NHCH$_2$CH$_2$), 2.77 (2 H, m, CH$_2$NH$_3$), 1.68 (4 H, m, CH$_2$CH$_3$), 1.42 (6 H, m, CH$_2$), 0.85 (3 H, t, CH$_3^A$), and 0.82 (3 H, t, CH$_3^B$). MS, *m/z*: 433 [*M* + H]$^+$; calc. for $C_{21}H_{32}N_6O_4$: 432.52.

Npc→Apc→NH(CH$_2$)$_4$NH←Apc←Npc (IVa) was synthesized by a procedure similar to that used for (**IV**) from (**III**) (10 mmol) and putrescine (5 mmol) (Merck); yield 46%; mp 182–185°C; R_f 0.91 (A) and 0.87 (B); ^{1}H NMR: 10.23 (2 H, s, ^{A}CONH^B), 8.23 (2 H, d, H5^A), 8.06 (2 H, t, CONHCH$_2$), 7.60 (2 H, d, H3^A), 7.25 (2 H, d, H5^B), 6.88 (2 H, d, H3^B), 4.40 (4 H, t, N^{A}CH$_2$), 4.26 (4 H, t, N^{B}CH$_2$), 3.23 (4 H, m, NHCH$_2$CH$_2$), 1.70 (8 H, m, CH$_2$CH$_3$), 1.54 (4 H, m, NHCH$_2$CH$_2$), 0.85 (6 H, t, CH$_3^A$), and 0.81 (3 H, t, CH$_3^B$); MS, *m/z*: 749.4 [*M* + H]$^+$; calc. for $C_{36}H_{48}N_{10}O_8$: 748.84.

Gly→Apc→Apc→NH(CH$_2$)$_5$NH←Apc←Apc←Gly (I). Compound (**IV**) (10.0 g, 13.1 mmol) was hydrogenated over Adams catalyst (2 g) in a mixture of ethanol (100 ml) and 6 M HCl (10 ml). The catalyst was filtered off, triethylamine (8.4 ml, 60 mmol) was added to the filtrate, and the mixture was evaporated. Anhydrous DMF (20 ml) was added to a mixture of Boc-Gly-OH (5.3 g, 30 mmol) and CDI (5.0 g). After 10 min, the resulting imidazolide solution was added to the amino component, kept for 20 h at 20°C, and precipitated with 0.5 M citric acid (200 ml). The precipitate was filtered and twice chromatographed on a Sephadex LH-20 column. The fraction containing (**I**) were combined and evaporated to give (**I**) as oil; yield 6.4 g. It was dissolved in TFA (50 ml), kept for 3 h at 25°C, evaporated, and twice chromatographed on a Sephadex LH-20 column followed by reversed-phase HPLC to give (**I**) bistrifluoroacetate (4.38 g, 32%); R_f 0.24 (A) and 0.75 (B). ^{1}H NMR: 10.67 (2 H, s, ^{A}CONH^B), 9.94 (2 H, s, CH$_2$CONH), 8.34 (6 H, m, H$_3$NCH$_2$), 7.98 (2 H, t, NHCH$_2$CH$_2$), 7.28 (2 H, d, H5^A), 7.24 (2 H, d, H5^B),

6.97 (2 H, d, H3^{A}), 6.88 (2 H, d, H3^{B}), 4.28 (8 H, t, NCH_2), 3.72 (4 H, m, $H_3NC\underline{H}_2CO$), 3.22 (4 H, m, $NHC\underline{H}_2CH_2$), 1.70 (8 H, m, $C\underline{H}_2CH_3$), 1.47 [6 H, m, $NHCH_2(C\underline{H}_2)_3$], and 0.82 (12 H, t, CH_3); MS, *m/z*: 817 [*M* + H]$^+$; calc. for $C_{41}H_{60}N_{12}O_6$: 816.99.

Gly—Apc—Apc—NH(CH$_2$)$_4$NH—Apc—Apc—Gly (Ia) was prepared from (**IVa**) (2.5 mmol) by a procedure similar to that in the synthesis of (**I**); yield 24%; R_f 0.15 (A) and 0.74 (B); ^{1}H NMR: 10.69 (2 H, s, $^{A}CONH^{B}$), 9.92 (2 H, s, $CH_2CON\underline{H}$), 8.24 (6 H, m, $\underline{H}_3NCH_2$), 8.00 (2 H, t, $N\underline{H}CH_2CH_2$), 7.27 (2 H, d, H5^{A}), 7.23 (2 H, d, H5^{B}), 6.98 (2 H, d, H3^{A}), 6.90 (2 H, d, H3^{B}), 4.27 (8 H, t, NCH_2), 3.74 (4 H, m, $NH_3C\underline{H}_2CO$), 3.21 (4 H, m, $NHC\underline{H}_2CH_2$), 1.68 (8 H, m, $C\underline{H}_2CH_3$), 1.55 [4 H, m, $NHCH_2(C\underline{H}_2)_2$], and 0.80 (12 H, t, CH_3); MS, *m/z*: 803 [*M* + H]$^+$; calc. for $C_{40}H_{58}N_{12}O_6$: 802.98.

Lys—Gly—Apc—Apc—NH(CH$_2$)$_5$NH—Apc—Apc—Gly—Lys (II). A solution of DCC (0.42 g, 2 mmol) in DMF (2 ml) was added to a mixture of Boc-Lys(Z)-OH (0.76 g, 2 mmol) and HOBt (Fluka, 0.33 g, 2 mmol) in DMF (5 ml), and the reaction mixture was kept for 30 min at 20°C. The precipitate was filtered, and the filtrate was added to (**I**) ditrifluoroacetate (0.52 g, 0.5 mmol). The reaction mixture was kept for 20 h at 20°C and loaded onto a Sephadex LH-20 column. After evaporation, the di-Boc-di-Z-derivative of (**II**) was obtained as oil (0.55 g), which was dissolved in a mixture of TFA (3 ml) and thioanisole (0.5 ml) (Fluka), kept for 1 h at 25°C, evaporated, and chromatographed twice on a Sephadex LH-20 column. After evaporation of the fractions containing di-Z-derivative of (**II**), it was obtained as oil (0.51 g). Z-protective groups were removed by hydrogenation over palladium black in methanol, and the target (**II**) was purified on Sephadex LH-20 column followed by reversed-phase HPLC; yield 0.43 g (80%); R_f 0.09 (A) and 0.46 (B); MS, *m/z*: 1073.6 [*M* + H]$^+$; calc. for $C_{53}H_{84}N_{16}O_8$: 1073.35.

Lys—Gly—Apc—Apc—NH(CH$_2$)$_4$NH—Apc—Apc—Gly—Lys (IIa) was similarly prepared from (**Ia**) in yield of 67%; MS, *m/z*: 1059.6 [*M* + H]$^+$; calc. for $C_{52}H_{82}N_{16}O_8$: 1059.32.

Npc—NH(CH$_2$)$_5$NH—Npc (VI). 1-Propyl-4-nitropyrrole-2-carboxylic acid [3] (20.0 g, 101 mmol) was refluxed with $SOCl_2$ (50 ml) and DMF (0.05 ml) for 40 min. The solution was evaporated, the residue was several times coevaporated with benzene and dissolved in ethanol (150 ml). Cadaverine (5.9 ml, 50 mmol) was added to the solution and then triethylamine (15 ml, 104 mmol) was added dropwise under vigorous stirring. After 2 h, the reaction mixture was cooled to 0°C, and the precipitate was filtered and recrystallized from ethanol to give 20.9 g (89%) of (**VI**); mp 204–206°C; R_f 0.94 (C) and 0.82 (D); ^{1}H NMR: 8.29 (2 H, t, CONH), 8.06 (2 H, d, H5), 7.34 (2 H, d, H3), 4.31 (4 H, t, $NC\underline{H}_2$), 3.18 (4 H, m, $CONHC\underline{H}_2$), 1.87–1.28 (10 H, m, CH_2), and 0.80 (6 H, t, CH_3); MS, *m/z*: 462.5 [*M* + H]$^+$; calc. for $C_{21}H_{30}N_6O_6$: 462.50.

Npc—NH(CH$_2$)$_4$NH—Npc (VIa) was prepared similarly; yield 76%; mp 217–220°C, R_f 0.93 (C) and 0.74 (D); ^{1}H NMR: 8.34 (2 H, t, CONH), 8.09 (2 H, d, H5), 7.36 (2 H, d, H3), 4.32 (4 H, t, NCH_2), 3.20 (4 H, m, $CONHC\underline{H}_2$), 1.8–1.4 (8 H, m, CH_2), and 0.79 (6 H, t, CH_3); MS, *m/z*: 449 [*M* + H]$^+$; calc. for $C_{20}H_{28}N_6O_6$: 448.47.

Z-Gly-Apc-OH (VII). Triethylamine (18.4 ml, 131 mmol) and the imidazolide solution obtained from Z-Gly-OH (13.6 g, 65 mmol) (Reanal), CDI (11.1 g, 65 mmol), and DMF (30 ml) were added for 10 min to a solution of 4-aminopyrrole-2-carbonyl chloride hydrochloride (13.4 g, 65.3 mmol) [23] in DMF (100 ml). The mixture was kept for 48 h at 20°C, evaporated, dissolved in water (300 ml), and acidified to pH 2 with conc. HCl. The precipitated oil was dissolved in ethyl acetate (1 l), washed with 1 M HCl (4 × 200 ml) and water, and dried. The solution was evaporated to the volume of 70 ml, cooled to 0°C; the resulting precipitate was filtered and washed with ethyl acetate and ether to give (**VII**); yield 18.8 g (80%); mp 153–154°C, R_f 0.96 (A) and 0.37 (D); ^{1}H NMR: 9.78 (1 H, s, $CH_2CON\underline{H}$), 7.43 (1 H, t, $CON\underline{H}CH_2$), 7.31 (5 H, s, C_6H_5), 7.28 (1 H, d, H5), 6.71 (1 H, d, H3), 5.03 (2 H, s, $C_6H_5C\underline{H}_2$), 4.19 (2 H, t, NCH_2), 3.74 (2 H, m, $NHC\underline{H}_2CO$), 1.66 (2 H, m, $C\underline{H}_2CH_3$), and 0.80 (3 H, t, CH_3); MS, *m/z*: 360 [*M* + H]$^+$; calc. for $C_{18}H_{21}N_3O_5$: 359.38.

Z-Gly—Apc—Apc—NH(CH$_2$)$_5$NH—Apc—Apc—Gly-Z (VIII). A solution of DCC (3.81 g, 18.5 mmol) in DMF (10 ml) was added to a solution of (**VII**) (6.47 g, 18 mmol) and HOBt (2.57 g, 19 mmol) in DMF (20 ml). The mixture was kept for 1.5 h at 20°C, the precipitate was filtered, and the filtrate was added to a solution of amino component, which was prepared by hydrogenation of (**VI**) (4.1 g, 8.9 mmol) over the Adams catalyst (1.0 g) in a mixture of ethanol (100 ml) and 6 M HCl (10 ml) followed by filtration and evaporation of the filtrate, in the mixture of triethylamine (8.4 ml, 60 mmol) and DMF (30 ml). The reaction mixture was kept for 24 h at 20°C, the precipitate was filtered, and the filtrate was evaporated. The residue was dissolved in chloroform (300 ml), washed with 1 M HCl, water, and 9% $NaHCO_3$ (2 × 100 ml), and dried. After evaporation of the solvent, the residue was dissolved in ethanol (150 ml) and allowed to stay for several days at 0°C. The resulting precipitate was filtered, washed with ethanol and ether to give 5.5 g (57%) of (**VIII**); mp 175–178°C, R_f 0.89 (A) and 0.53 (D); ^{1}H NMR: 10.71 (2 H, s, $^{A}CONH^{B}$), 9.76 (2 H, s, $CH_2CON\underline{H}^{A}$), 7.91 (2 H, t, OCONH), 7.31 (10 H, s, C_6H_5), 7.15 (4 H, m, H5$^{A, B}$), 6.85 (2 H, d, H3^{A}), 6.80

(2 H, d, H3^{B}), 5.04 (4 H, s, $C_6H_5C\underline{H}_2$), 4.20 (8 H, t, NCH_2), 3.76 (4 H, d, $NHC\underline{H}_2CO$), 1.79–1.33 (14 H, m, $C\underline{H}_2CH_3$ + $NHCH_2C\underline{H}_2C\underline{H}_2C\underline{H}_2$), and 0.79 (12 H, t, CH_3); MS, *m/z*: 1085 $[M + H]^+$; calc. for $C_{57}H_{72}N_{12}O_{10}$: 1085.28.

Z-Gly→Apc→Apc→NH(CH$_2$)$_4$NH←Apc←Apc←Gly-Z (VIIIa) was similarly synthesized from (**VIa**) (2 mmol); yield 1.8 g (83%), R_f 0.85 (A) and 0.47 (D).

Z-groups were removed from (**VIII**) and (**VIIIa**) by hydrogenation over palladium black, and the resulting (**I**) and (**Ia**) were identical to those prepared according to Scheme 1.

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