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QUARTERLY

# New carbocyclic analogues of netropsin: Synthesis and inhibition of topoisomerases★

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**A series of carbocyclic analogues of netropsin were synthesized and evaluated for their capacity to inhibit human topoisomerases I and II *in vitro*. The compounds are oligopeptides containing 1,4-di- and 1,2,5-trisubstituted benzene rings and unsubstituted N-terminal $NH_2$ groups. Compounds 4–7 consist of two netropsin-like units linked by aliphatic (tetra- and hexamethylene) chains. In the topoisomerase I and II assay, the relaxation of pBR322 plasmid was inhibited by compounds 4–7 at 100 $\mu$M concentration.**

The investigation of biological consequences of DNA modifications, caused by compounds endowed with the capacity to interact directly with DNA, provides vital information on the general molecular process of recognition of DNA by DNA-interacting antitumour agents. Many agents act by binding within the minor groove of double helical B-DNA, interfering with both replication and transcription. The earliest characterization of DNA minor-groove binding was reported in studies of antibiotics distamycin and netropsin [1, 2].

The exact mechanisms by which these compounds act *in vivo* are still elusive. One of the possibilities is that they might regulate gene expression by altering the binding of important regulatory proteins to their natural target sequences. A large number of DNA-binding agents (bis-netropsin, bisbenzamide Hoechst 33258) interfere with the catalytic activities of topoisomerases [3, 4] and, in some cases, this effect is believed to be a primary determinant of the cytotoxic properties of the drug. Topoisomerases are nuclear enzymes that regulate topological and conformational changes in DNA, critical to cellular processes such as replication, transcription, chromosome segregation and mitosis [5]. Moreover,

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they are extremely important in the area of therapy for human cancers and viral diseases, as topoisomerases are privileged targets for a variety of antineoplastic and antiviral agents [5].

As a part of an ongoing rational drug design programme aiming at development of carbocyclic analogues of netropsin as potential anticancer drugs, seven novel compounds **1–7** (Fig. 1) were synthesized and evaluated as inhibitors of topoisomerase I and II.

## MATERIALS AND METHODS

***Reagents and materials.*** Five new compounds **1, 3, 4, 6** and **7** were synthesized as hydrochlorides with high yields by standard chemical transformations according to the reaction sequences shown in Figs. 2 and 3. The required starting materials **I**, **VII, VIII** and **IX** were prepared according to the methods worked out in our laboratory for compound **2** [6], and **5** [7] from 2-hydroxy-5-phenylazobenzoic acid, obtained by the method described by Polaczkowa [8]. The synthesis and complete physicochemical characterisation of the designed compounds will be reported elsewhere. Purity of these compounds was verified by NMR and elementary analysis.

4-Nitrobenzoyl chloride **(III)** was obtained from Merck. Topoisomerase I, pBR322 plasmid DNA, proteinase K and ethidium bromide were purchased from Sigma Chemical Co. (U.S.A.) and topoisomerase II from Amersham Pharmacia Biotech.

***Assay of DNA relaxation by topoisomerases I and II.*** pBR322 plasmid DNA

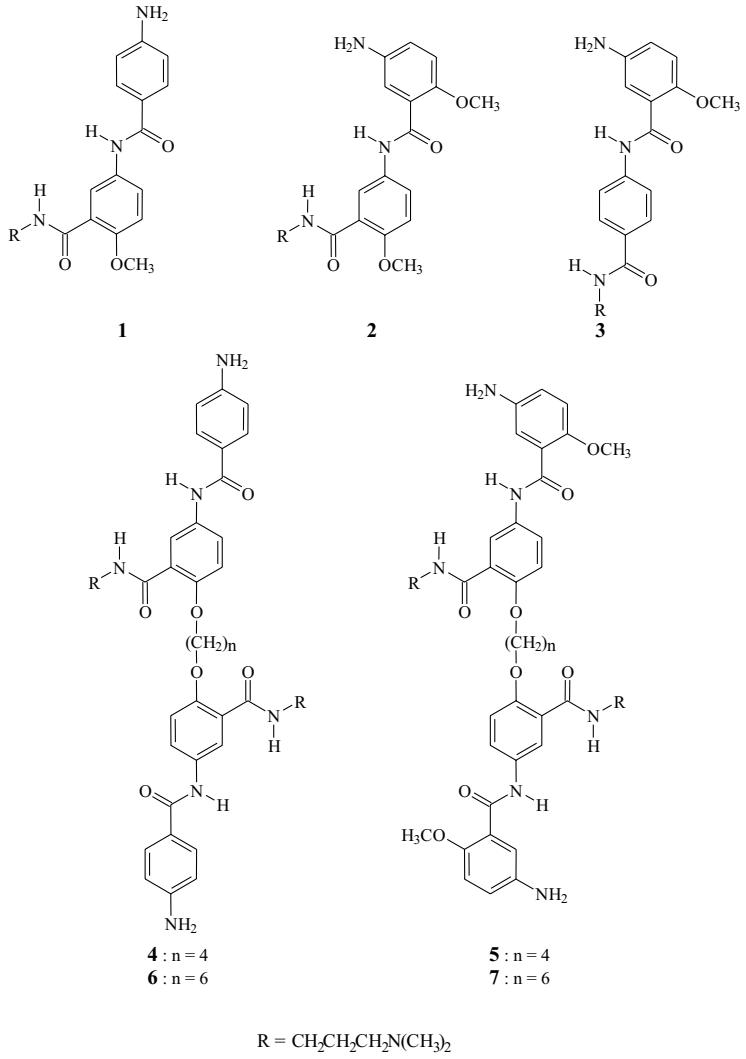


**Figure 1. Structure of compounds 1–7.**

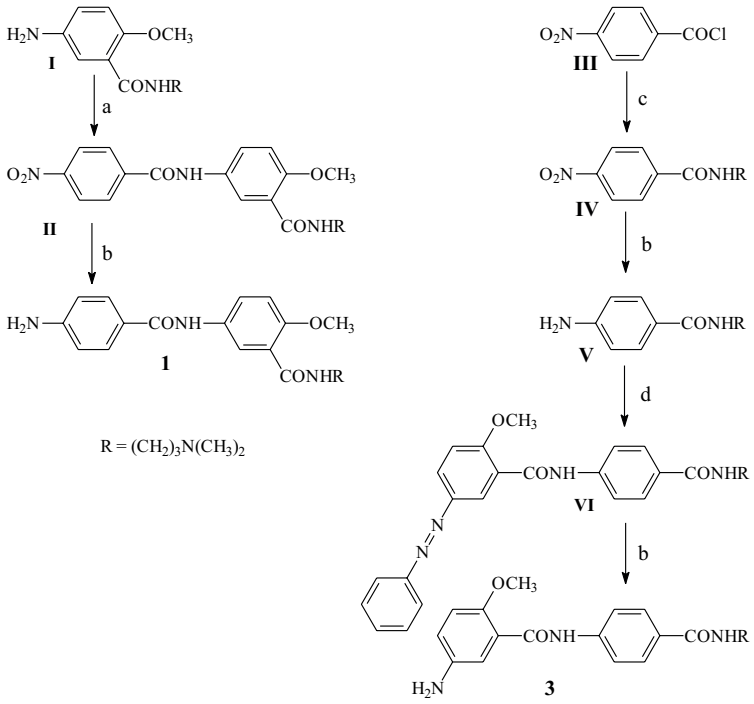


**Figure 2. Synthesis of compounds 1 and 3.**

Reagents and conditions: a) 4-nitrobenzoyl chloride **(III)**, $Py/CH_2Cl_2$, 4-(dimethylamino)pyridine (DMAP); b) $H_2$/Pd, MeOH, HCl; c) 3-dimethylamino-1-propylamine, $CH_2Cl_2$; d) 2-methxy-5-phenylazobenzoyl chloride **(VII)**, $Py/CH_2Cl_2$, DMAP.

(0.25 $\mu$g) was incubated with 3 units of topoisomerase I (reaction buffer: 50 mM Tris/HCl, pH 7.9, 1 mM EDTA, 0.5 M NaCl, 1 mM dithiothreitol) or topoisomerase II (reaction buffer: 10 mM Tris/HCl, pH 7.9, 1 mM ATP, 50 mM KCl, 5 mM $MgCl_2$, 50 mM NaCl,

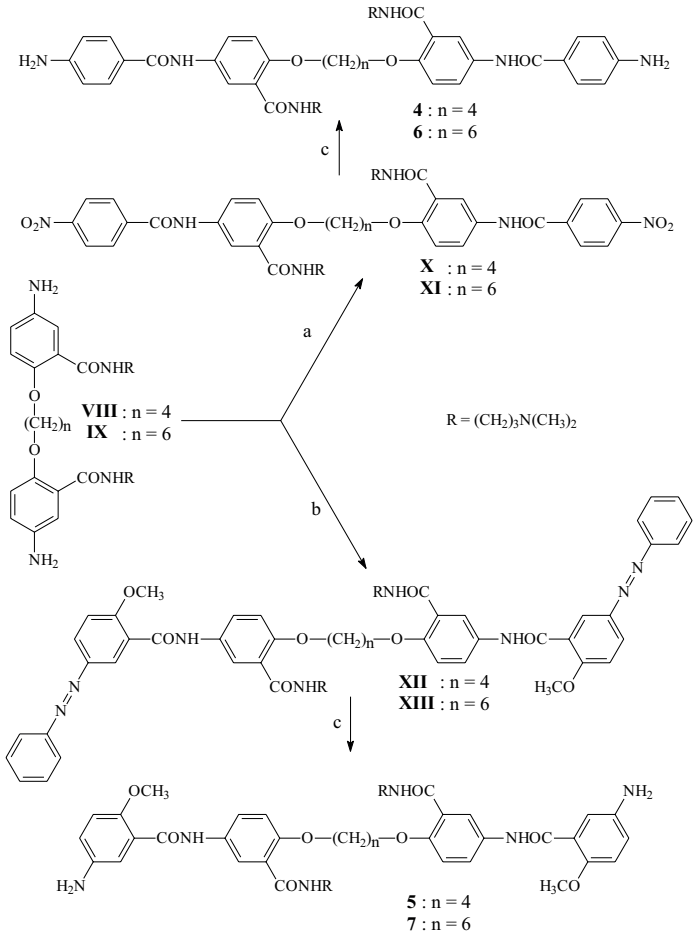


**Figure 3. Synthesis of compounds 4–7.**

Reagents and conditions: a) 4-nitrobenzoyl chloride **(III)**, $Py/CH_2Cl_2$, 4-(dimethylamino)pyridine (DMAP); b) 2-methoxy-5-phenylazobenzoyl chloride **(VII)**, $Py/CH_2Cl_2$, DMAP; c) $H_2$/Pd, MeOH, HCl.

0.1 mM EDTA, and 15 $\mu$g/ml bovine serum albumin) in the presence of the tested compounds at the concentration of 100 $\mu$M. The mixture was incubated at 37°C for 1 h and the reaction was terminated by addition of 2 $\mu$l of 10% SDS and 2 $\mu$l of proteinase K (1 mg/ml). The reaction mixture was subjected to electrophoresis on 0.8% agarose gel containing 0.5 $\mu$g/ml ethidium bromide in TBE buffer (90 mM Tris/borate and 2 mM EDTA). The gels were stained with ethidium bromide and photographed under UV light.

## RESULTS AND DISCUSSION

Structural studies on various minor groove binders complexed with different DNA sequences have proven useful in gaining understanding of the principles of selectivity in minor groove recognition. Three main factors contribute to the stability between specific sequences of DNA and lexitropsins – analogues of netropsin and distamycin: 1) hydrogen bonding between the amide NH of the drug and DNA bases; 2) an overall shape complementarity resulting in close ligand–DNA van der Waals interactions; 3) electrostatic attractions between the anionic DNA and the cationic drugs [9]. The possibility of rational design of sequence-specific DNA binding molecules stimulated the synthesis of several classes of new compounds of potential use in cancer chemotherapy. The replacement of *N*-methylpyrrole rings of netropsin or distamycin with other heterocyclic or carbocyclic moieties [10–12], the covalent linkage of two lexitropsin molecules (the motif of bis- and hairpin-lexitropsin) [13, 14] and the coupling with agents of known biological activity [15–17] produced a large number of sequence-specific DNA binding molecules with a wide range of activities [9].

Compounds **1–7** are oligopeptides in which *p*-aminobenzoic and 5-amino-2-methoxybenzoic acids are coupled by peptide bonds. 3-Dimethylamino-1-propylamine group of compounds **1–7** (p$K_a$ of about 9.3) would be protonated at physiological pH of 7.4 to provide favourable electrostatic attraction to neg-

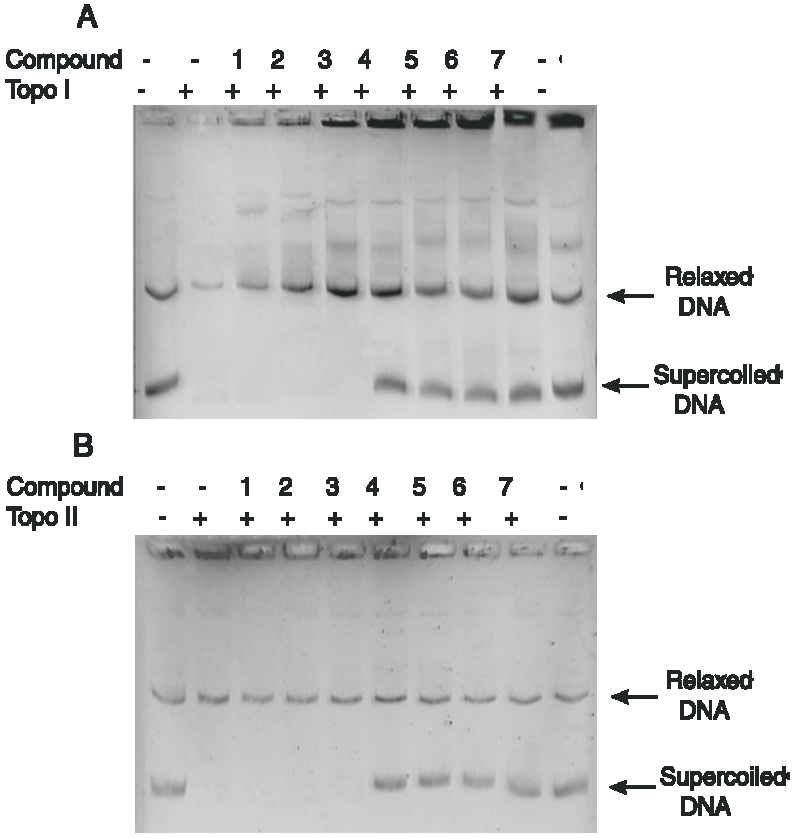


**Figure 4. Effect of topoisomerase-mediated DNA relaxation in the presence of compounds 1–7: A) topoisomerase I, B) topoisomerase II.**

ative electrostatic potential of the DNA. Furthermore, the sequence selectivity of the dimethylamino moiety has been shown to be similar to that of the amidinium group of netropsin [9]. Compounds **1**–**3** have the same structural motif of two benzene units connected by an amide bond, giving the molecules a crescent shape. Ligands **1** and **3** differ in the sequence of di- and tri-substituted benzene rings. Compound **2** differs from **1** by the location of N-terminal $NH_2$ group and its one aromatic ring being substituted with methoxy group in *ortho* position. To enhance the binding of carbocyclic analogues of netropsin to DNA, we synthesized compounds **4**–**7** linking both compounds **1** and **2** with aliphatic tetra- and hexamethylene chains (Fig. 1). The incorporation of the flexible linker permits a better fit and antiparallel orientation of subunits of **4**–**7** within the minor groove [18].

The replacement of heterocyclic rings by carbocyclic moieties yields lexitropsins which, in comparison with distamycin, show lower toxicity and increased antibacterial, antiviral and antitumour activity [19]. Compound **2** and its derivative with a chlorambucil moiety showed antiproliferative and cytotoxic effects on the cultured breast cancer MCF-7 cells [20, 21]. These compounds bind to AT sequences more weakly than the extensively studied minor-groove binders, such as distamycin and netropsin. Molecular modelling shows that compound **2** is actually isohelical with the DNA minor groove. From the energetic analysis it appears that Van der Waals terms are more important than electrostatic interactions and specific hydrogen bonds in stabilizing of **2**–d$(CGCGAATTCGCG)_2$ complex [22]. This view was supported by a study on a series of bis-benzimidazole–DNA complexes, which showed that an increase in the number of charged groups did not significantly affect the binding ability, whereas with increasing number of contacts with the minor groove walls the affinity became increased [23].

To identify the biochemical target(s) of compounds **1**–**7**, we investigate their action on topoisomerases using a gel electrophoresis assay. Purified topoisomerases I and II were incubated with increasing concentrations of **1**–**7** in the presence of supercoiled plasmid DNA pBR322, and the products were subjected to electrophoresis in the presence of ethidium bromide to separate closed and open circular DNA. Figure 4A shows the result of analysis of topoisomers of pBR322 DNA resulting from topoisomerase I action. The superhelical plasmid (lane 1) was relaxed by topoisomerase I (lane 2). Compounds **1**–**3** at the concentration of 100 $\mu$M had no effect on the ability of topoisomerase I to transform supercoiled DNA into topoisomer forms of relaxed DNA. However, the addition of **4**–**7** at the concentration of 100 $\mu$M caused the appearance of superhelices that looked similar to the intact plasmid (compare lane 6 with lane 1). Figure 4B shows the action of compounds **1**–**7** and topoisomerase II. Compounds **1**–**3** did not inhibit the topoisomerase II mediated relaxation of supercoiled DNA. In this case, the relaxation of DNA was inhibited by compounds **4**–**7** at the concentration of 100 $\mu$M.

The fact that compounds **4**–**7** are more potent than **1**–**3** indicates that inhibition of topoisomerases is selective and sensitive to the number of repeating benzene carboxamide units. A minimum of four benzene carboxamide units (compounds **4**–**7**) are necessary for the inhibition of topoisomerases. Under identical conditions, complete inhibition of DNA cleavage was obtained using 2 $\mu$M camptothecin or 10 $\mu$M etoposide, drugs which are potent inhibitors of topoisomerase I and topoisomerase II, respectively [24, 25].

Complete characterisation of biological properties (anticancer activity, DNA binding) of the described compounds requires further studies which could explain the results reported here. We suppose that these carbocyclic analogues of netropsin could be

used as drug carriers or will show antitumour properties.

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