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Synthesis, DNA Binding, Topoisomerases Inhibition and Cytotoxic Properties of 4-Arylcarboxamidopyrrolo-2-carboxyanilides

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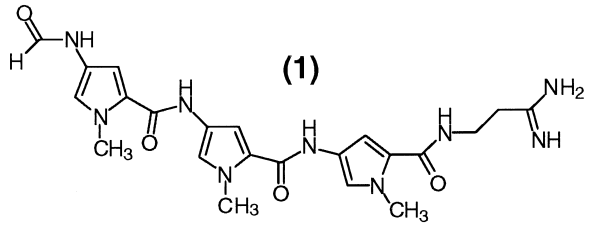
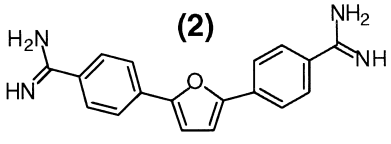
Abstract—Three 4-arylcarboxamidopyrrolo-2-carboxyanilides bearing different substituents on the pyrrole nitrogen were synthesized and evaluated for their capacities to bind to specific sequences within the minor groove of DNA and to inhibit human topoisomerases I and II in vitro. The cytotoxicity of the drugs correlates with their DNA binding affinities. The two drugs bearing a *N*-methyl or *N*-benzyl pyrrole stabilize topoisomerase I-DNA complexes.

The pyrrole-amidine antibiotics netropsin and distamycin (**1**) represent prototypic DNA minor groove binders endowed with a pronounced selectivity for AT-rich sequences but are weakly cytotoxic. A vast number of lexitropsins analogues and conjugates targeting specific sequences in DNA have been designed.[1] Several potent antitumor drugs have been obtained, such as the distamycin–nitrogen mustard conjugate tallimustine which is undergoing clinical trials as an anticancer agent.[1] AT-specific recognition also occurs with furamidine (**2**) and related diphenylfuran derivatives which present interesting therapeutic profiles, in particular as anti-*Pneumocystis* agents.[2,3]

In addition to interacting with specific sequences in DNA, minor groove binders can interfere with the catalytic activities of nuclear enzymes involved in the regulation of DNA topology in cells. Topoisomerases I and II are the primary targets of many anticancer drugs including the anthracyclines and camptothecins.[4,5] Certain DNA minor groove binding agents related to Hoechst 33342 or bifunctional netropsin analogues act as poisons for topoisomerase I.[6–8] This ubiquitous enzyme is considered a valid target for the development of antitumor agents.

These considerations prompted us to design a new series of potential DNA minor groove binder inhibitors of topoisomerases. We developed the 4-arylcarboxamidopyrrolo-2-carboxyanilide skeleton which combines the structural features of distamycin (**1**) and furamidine (**2**). The amidine side chains were replaced with more stable





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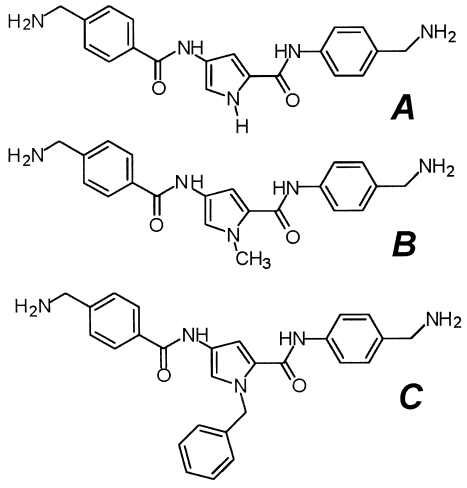
primary amine-containing chains. The central pyrrole ring was substituted with either a *N*-methyl group as found in distamycin or netropsin or with a more bulky *N*-benzyl group.

Here we report the synthesis of the drugs ***A***, ***B*** and ***C*** and their DNA biding affinity and binding mode characterized by a combination of spectroscopic approaches including absorption, fluorescence, circular dichroism and electric linear dichroism. A relaxation assay employing supercoiled plasmid DNA was used to evaluate the effects of the drugs on human topoisomerases.



Synthesis

Compounds ***A***, ***B*** and ***C***[9] were prepared from three differently substituted pyrroles **3**, **4** and **5**. The pyrrole heterocycle was obtained by nitration of mucobromic acid **1** (commercially available) with sodium nitrite in ethyl alcohol at 54 °C, followed by cyclization of the nitromalonaldehyde carbanion **2** with glycine methyl ester hydrochloride in sodium hydroxide and methyl alcohol at 50 °C.[10] *N*-Substituted pyrroles **4** and **5** were obtained from pyrrole **3** which was alkylated by iodomethane or benzyl bromide in DMF at room temperature (Scheme 1). The *N*-methylpyrrole **4** can also be obtained from the nitration of pyrrole-2-carboxylic acid, as previously described.[11]

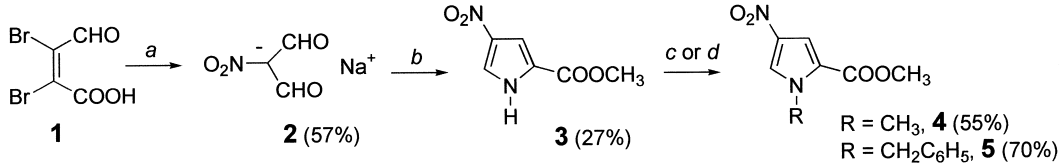
Compound **9** was prepared from 4-nitrobenzonitrile **6** (Scheme 2).[12] The nitrile group was reduced (**7**) with borane at reflux in THF prior to protection of the resulting amino group with di-*tert*-butyldicarbonate in dioxane and water at pH 10 (**8**).[13] Then, the nitro group of **8** was reduced in the presence of Pd/C.

Esters **3**–**5** were saponified overnight at room temperature with sodium hydroxide in water and THF to afford **10**,[14] **11**[14] and **12**, and then coupled with the aniline derivative **9** using PyBOP as a coupling agent in DMF.

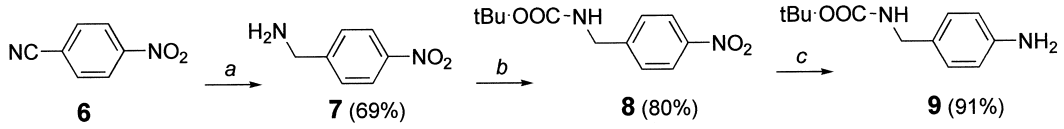
Catalytic hydrogenation of the resulting amides (with Pd/C at room temperature for **13** and at atmospheric pressure and Raney nickel for **14** and **15** at 60 °C and 30 bars) gave amines **16**, **17** and **18** which are unstable and therefore were immediately coupled with **19** with PyBOP in *N*-methylpyrrolidinone. 4-*tert*-Butoxycarbonylamino)benzoic acid **19**[15] was obtained from 4-(aminomethyl)benzoic acid (commercially available) by reaction with di-*tert*-butyldicarbonate in a water/dioxane mixture containing NaOH. Finally, protected amino groups of compounds **20**–**22** were released in the presence of TFA to afford 4-(4-aminomethylphenylcarboxamido)-2-(4-aminomethyl)-1*H*-pyrrolecarboxamide (***A***), its *N*-methyl (***B***) and *N*-benzyl (***C***) analogues, respectively (Scheme 3).

DNA Binding Affinity

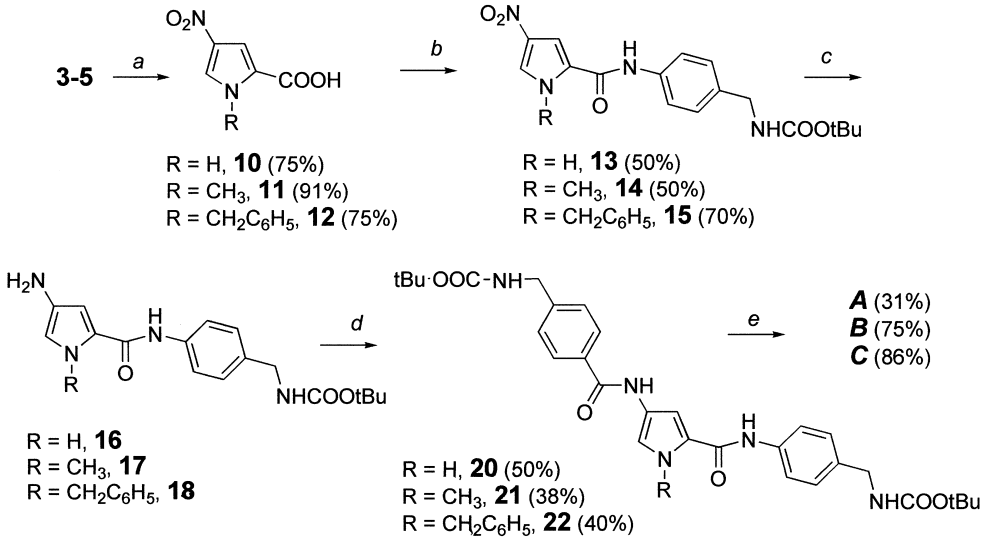
Complex formation between the drugs ***A***, ***B***, ***C*** and DNA results in a significant increase in the T_m values of helix-to-coil transition (Fig. 1). A 31 °C increase in the T_m of poly(dA-dT)·(dA-dT) is observed for compounds ***A*** and ***B*** whereas the ΔT_m value with ***C*** reaches 23 °C. With calf thymus DNA, under similar conditions, the measured ΔT_m values ($T_m^{\text{drug-DNA complex}}$-$T_m^{\text{DNA}}$) were 16.3, 11.6 and 6.8 °C with ***A***, ***B*** and ***C*** respectively. The stabilisation of the duplex structure is weaker with **C** than with ***A*** and ***B***. DNA binding affinity constants were determined from fluorescence titration experiments. As indicated in Table 1, the K_a value for compound ***C*** is about 3 times lower than that calculated for ***A***. The *N*-benzyl group reduces slightly the strength of the drug-DNA interaction.



Scheme 1. Reagents and conditions: (a) $NaNO_2$, EtOH, 54 °C; (b) $HCl,NH_2CH_2COOCH_3$, 50 °C, NaOH, MeOH; (c) NaH, CH_3I, DMF; (d) NaH, $BrCH_2\Phi$, DMF.



Scheme 2. Reagents and conditions: (a) BH_3 Me_2S, THF; (b) Boc_2O, NaOH, dioxane/water; (c) H_2, Pd/C, MeOH.



Scheme 3. Reagents and conditions: (a) NaOH, H_2O/THF then HCl; (b) **9**, PyBOP, DIPEA, DMF; (c) H_2, Pd/C or Raney nickel, MeOH; (d) **19**, PyBOP, DIPEA, *N*-methylpyrrolidinone; (e) TFA, CH_2Cl_2 then $NaHCO_3$, H_2O.

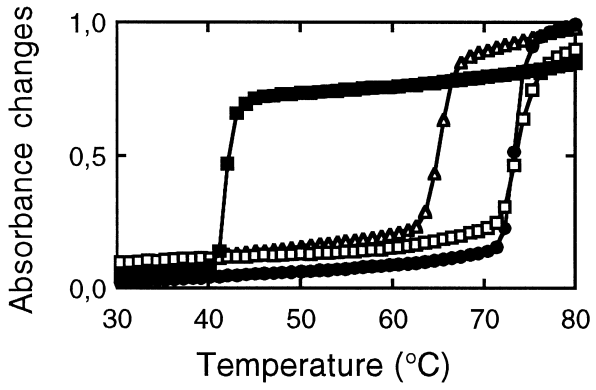


Figure 1. Thermal denaturation curves for poly(dA-dT)·(dA-dT) in the absence (■) and presence of ***A*** (□), ***B*** (●) and ***C*** (△) (2 μM each). T_m measurements were performed in BPE buffer pH 7.1 (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA) using 20 μM DNA in 3 mL quartz cuvettes at 260 nm with a heating rate of 1 °C/min. Each drug concentration was tested in triplicate. The T_m values were obtained from first-derivative plots.

Table 1. Cytotoxicity and DNA binding affinity

Compounds	IC_{50} (μM)	K_a (10^5 M^{-1})
A	1.7 ± 0.3	9.5
B	5.4 ± 0.3	6.8
C	8.2 ± 0.6	3.4

DNA Binding Mode

Figure 2 (left) displays the circular dichroism (CD) spectrum of ***B*** bound to DNA. The positive CD band around 310–340 nm reflects the orientation of the drug in the minor groove of the double helix.[16] The geometry of the drug–DNA complexes was investigated further using electric linear dichroism (ELD).[17] Figure 2 (right) shows the dependence of the reduced dichroism ΔA/A on the wavelength for compound ***B***. The ELD signal is always positive in sign in the 300–390 nm absorption band, consistent with an inclination of the chromophore at about 45° with respect to the orientation axis of the DNA molecules, as expected for a minor groove binder.[17] The CD and ELD measurements provide strong evidences that the 4-arylcarboxamidopyrrolo-2-carboxyanilides bind into the minor groove of DNA double helix.

Sequence Specificity

DNase I footprinting experiments were carried out with the 160 bp *tyr*T fragment which has been previously used to map distamycin and furamidine binding sites.[18–20] The gel in Figure 3 shows clearly that the three drugs recognize preferentially AT-rich sequences. The footprints are less pronounced with ***C*** than with ***A*** or ***B*** (compare the 2 μM lanes) but however, the introduction of the *N*-benzyl group on the central pyrrole does not abolish the capacity of the drug to bind selectively to AT sites. Four well-resolved footprints encompassing the sequences 5′-AAAT, 5′-TAAA, 5′-AAAAAT and 5′-TAATTTT can be detected using 5 μM drugs. Regions protected from DNase I cleavage by the drugs are confined to the same AT-rich sequences as those detected with distamycin and furamidine.[18]

Topoisomerases Inhibition

Closed circular DNA was treated with human topoisomerase I or II in the presence of the test drugs at 10 or 50 μM. DNA samples resolved in 1% agarose gels containing ethidium bromide. In both cases, supercoiled plasmid DNA was fully relaxed by the enzyme (I or II) in the absence of the drug. As shown in Figure 4 (right), a band of linear DNA (form III) could be seen with

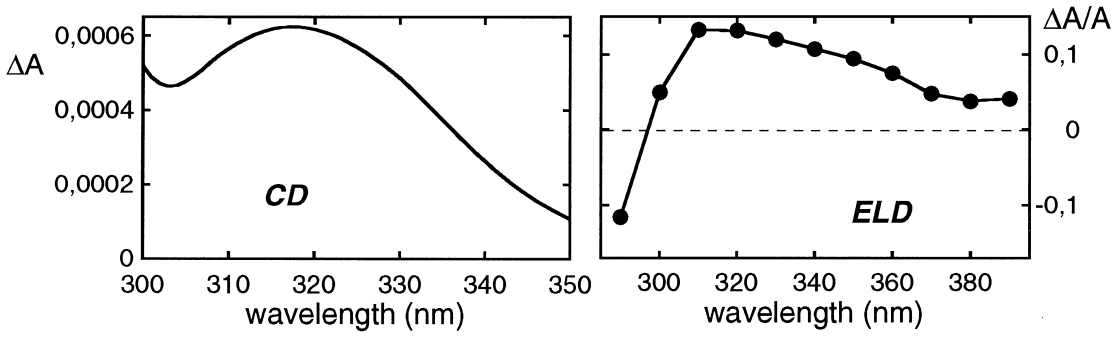


Figure 2. (CD) Circular dichroism and (ELD) electric linear dichroism spectra of compound ***B*** bound to calf thymus DNA at a P/D ratio of 20, in 1 mM sodium cacodylate buffer pH 7.0.

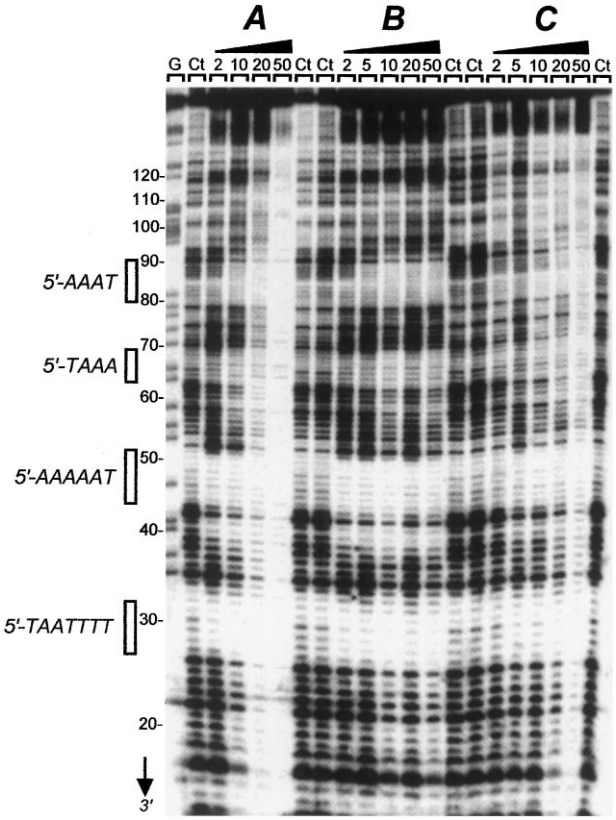


Figure 3. DNase I footprinting of ***A***, ***B*** and ***C*** bound to the *tyrT* DNA fragment. The DNA was 3′-end labeled with [α-^{32}P]dATP in the presence of AMV reverse transcriptase. The drug concentration (μM) is shown at the top of the appropriate gel lanes. The track labelled "Ct" contained no drug. The track labeled "G" represents a dimethylsulphate-piperidine marker specific for guanines. Numbers on the side of the gels refer to the numbering scheme of the fragment. DNase I cleavage products were resuspended in 5 μL 80% formamide containing 10 mM EDTA and 0.1% tracking dyes. Samples were heated to 90 °C for 4 min and then chilled in an ice-bath just before being loaded on a sequencing gel (8% polyacrylamide, 7 M urea). The AT-rich sequences protected from cleavage are indicated.

etoposide which is a well known topoisomerase II poison. No such band of linear DNA was detected with the 4-arylcarboxamidopyrrolo-2-carboxyanilides. They have no effects on topoisomerase II.

The same relaxation assay was used to investigate the effects of the drugs on topoisomerase I (Fig. 4, left). In the presence of compound ***C*** at 10 or 50 μM, the intensity of the band corresponding to the nicked form II of DNA has increased significantly. This effect, observed with camptothecin (positive control), reflects the stabilization of topoisomerase I–DNA cleavable complexes. Compound ***A*** has no effect on topoisomerase I whereas ***C*** and to a lower extent ***B*** behave as specific topoisomerase I inhibitors trapping the covalent DNA–topoisomerase I complexes.

Cytotoxicity

The compounds were evaluated for their antiproliferative activities using human HL60 leukemia cells.[21] The results expressed as IC_{50} values (concentration reducing by 50% the cell proliferation) are reported in the Table 1. There is apparently no correlation between the IC_{50} values and the effects of the drug on topoisomerase I but interestingly, a link can be established with the DNA binding data. The fact that the *N*-benzyl compound ***C*** is about 4 times less cytotoxic than compound ***A*** likely account for its reduced capacity to interact with DNA.

Conclusion

4-Arylcarboxamidopyrrolo-2-carboxyanilides represent a new series of sequence-selective minor groove binders. The substitution of the pyrrole nitrogen with a benzyl group slightly reduces the DNA interaction but however reinforces the capacity of the drug to inhibit topoisomerase I. The benzyl group might serve as a hook to anchor the enzyme close to the cleavage sites on DNA. Such a behavior has been reported with the DNA minor groove binding antitumor drug ecteinascidin[22,23] which is also an inhibitor of topoisomerase I.[24] The benzyl group of compound ***C*** likely behaves as the external C domain of ecteinascidin which protrudes outside the DNA double helix. The design of DNA minor groove binders equipped with such a protein-trapping domain may provide a new avenue for the design of antitumor drugs.

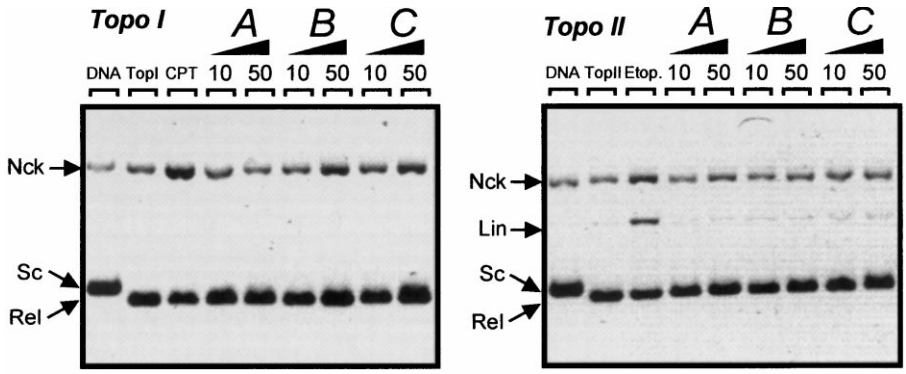


Figure 4. Effect of ***A***, ***B*** and ***C*** on the relaxation of supercoiled plasmid DNA by topoisomerase I or II. The DNA (0.5 μg) was incubated with 4 units topoisomerase in the absence (lane TopI/II) or presence of drug at 10 or 50 μM. Etoposide (Etop.) and camptothecin (CPT) were used at 50 μM. Reactions were stopped with SDS and proteinase K. DNA samples were electrophoresed on agarose gel containing ethidium bromide. Nck, nicked; Lin, linear; Rel, relaxed; Sc, supercoiled.

Acknowledgements

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9. **A**: mp >260 °C; IR (KBr) 3500–3250, 1645, 1640, 1600; ^{1}H NMR (300 MHz; TFA) δ 4.45 (sl, 2, CH_2), 4.54 (sl, 2, CH_2), 7.80 (m, 11, aromatics); MS (CI) for $C_{20}H_{21}N_5O_2$, $2H_2O$ (MH^-) calcd 362, found 362. Anal. ($C_{59.73}H_{6.10}N_{17.01}O_{17.16}$) CHN. **B**: mp 185 °C; IR (KBr) 3310, 1650, 1610; ^{1}H NMR (300 MHz; TFA) δ 4.03 (s, 3, CH_3), 4.46 (sl, 2, CH_2), 4.54 (sl, 2, CH_2), 7.56–06 (m, 10, aromatics); MS (EI) for $C_{21}H_{23}N_5O_2$, $2H_2O$ (M^+) calcd 377, found 377. Anal. ($C_{61.25}H_{6.41}N_{16.75}O_{15.59}$) CHN. **C**: mp 99–101 °C; IR (KBr) 3420, 3030, 1675, 1605; ^{1}H NMR (300 MHz; TFA) δ 3.92 (s, 2, CH_2), 4.13 (s, 2, CH_2), 5.63 (sl, 2, CH_2), 7.15–34 (m, 5, aromatics), 7.28 (s, 1, H_5), 7.38 (d, J=8.37 Hz, 2, $H_{3''}$), 7.54 (s, 1, H_3), 7.60 (d, J=7.87 Hz, 2, $H_{3'}$), 7.74 (d, J=8.37 Hz, 2, $H_{2''}$), 8.00 (d, J=7.87 Hz, 2, $H_{2'}$), 8.20 (sl, 2, NH_2), 8.38 (sl, 2, NH_2), 10.09 (s, 1, NH), 10.51 (s, 1, NH); MS (EI) for $C_{27}H_{27}N_5O_2$ (M^+) calcd 453, found 453. Anal. ($C_{70.00}H_{5.85}N_{15.30}O_{8.85}$) CHN.
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