Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Medicinal Chemistry 40 (2005) 1206–1213
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
www.elsevier.com/locate/ejmech

Original article

Synthesis of 2,6-diphenylpyrazine derivatives and their DNA binding and cytotoxic properties

Nathalie Dias [b], Ulrich Jacquemard [a], Brigitte Baldeyrou [b], Amélie Lansiaux [b], Jean-François Goossens [c], Christian Bailly [b,1], Sylvain Routier [a,*], Jean-Yves Mérour [a]

[a] *Institut de Chimie Organique et Analytique, UMR CNRS 6005, Université d'Orléans, B.P. 6759, 45067 Orléans cedex 2, France*
[b] *Inserm U-524, Centre Oscar Lambret, IRCL, Place de Verdun, 59045 Lille, France*
[c] *Laboratoire de Chimie Analytique, Faculté de Pharmacie, Université de Lille 2, 59006 Lille, France*

Received 6 April 2005; received in revised form 22 June 2005; accepted 1 July 2005

Available online 08 September 2005

Abstract

A series of 2,6-diphenylpyrazine derivatives was synthesized from 2,6-dichloropyrazine and 4-methoxyphenylboronic acid using palladium(0) as catalyst in a Suzuki methodology. After deprotection of the hydroxyl, alkylation reactions with different halides afforded compounds **5**–**8** bearing hydrophilic chains. DNA binding and cytotoxic properties were investigated. Compound **11** bearing imidazoline terminal groups was found to be a potent AT-specific DNA minor groove binder but there was no relationship between DNA interaction and cytotoxicity. However, in all cases the incorporation of the pyrazine ring was found to promote the cytotoxicity of the molecules compared to the corresponding pyridine analogues, previously synthesized.
© 2005 Elsevier SAS. All rights reserved.

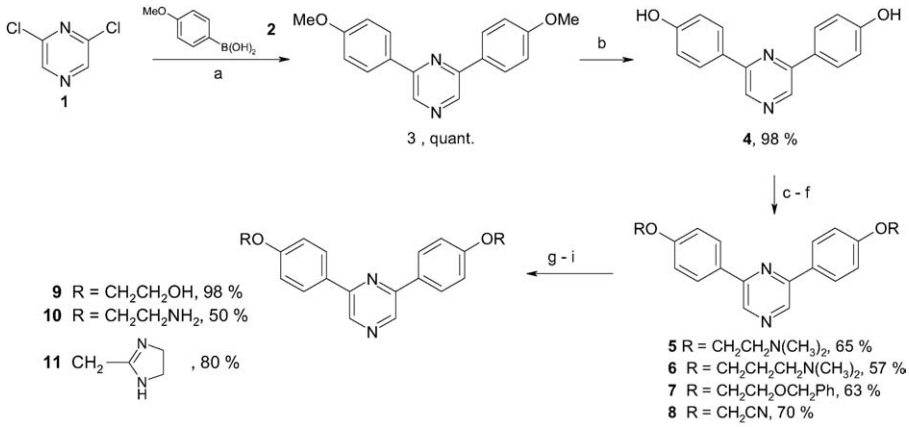
Keywords: Pyrazine derivatives; Cytotoxicity; DNA binding; Anticancer agents

1. Introduction

DNA remains an attractive target for the design of antitumor and antiparasitic agents [1,2]. For more than 20 years, medicinal chemists have elaborated small molecules capable of reading the genetic information embedded in the DNA base pairs. Successful strategies have been built in four broad, structurally distinct series of molecules that bind to the minor groove of DNA: (i) hairpin polyamide essentially composed of pyrrole- and imidazole-carboxamide units that remarkably sense the minor groove surface of DNA to distinguish almost all types of A-T and G-C base pair combinations [3,4], (ii) bis and ter-benzimidazoles showing tight binding to DNA and in some cases inhibition of topoisomerase I coupled with potent antitumor activities [5,6], (iii) pyrrolobenzodiazepine and cyclopropylpyrroloindole DNA damaging agents [7,8] and (iv) bis-amidines derived from the diphenylfuran compound furamidine which is a highly promising anti-infectious agent and a potent AT-selective DNA binder [9]. This latter bis-amidine series has been considerably expanded over the past 10 years to provide a repertoire of potent DNA recognition molecules all possessing an extended unfused aromatic system. This approach has yield a considerable variety of therapeutically active compounds useful to combat various types of infectious diseases, such as African trypanosomiasis and *Pneumocystis carinii* pneumonia for examples [9]. All these molecules represent a rich source of information from which we can design novel categories of DNA recognition compounds. With this idea in mind, we have recently reported the synthesis of a series of bis-phenylpyridine derivatives substituted with different side chains, neutral or cationic [10]. A few cytotoxic molecules were identified but their antiproliferative activity did not correlate with DNA binding. In an extension of this work, we report here the synthesis of a follow up series of molecules for which the pyridine central ring has been replaced with a pyrazine ring. Eight molecules (Scheme 1) have been prepared and the DNA binding and cytotoxic properties were investigated for six of them which could be directly compared to their pyridinic analogs.

* Corresponding author.
E-mail address: sylvain.routier@univ-orleans.fr (S. Routier).
[1] Present address: Centre de Recherche en Oncologie Expérimentale, Institut de Recherche Pierre Fabre, 3, rue des satellites, 31400 Toulouse, France.

0223-5234/$ - see front matter © 2005 Elsevier SAS. All rights reserved.
doi:10.1016/j.ejmech.2005.07.003



Scheme 1. **a)** 4-Methoxyphenylboronic acid **2** (2.5 eq.), $Pd(PPh_3)_4$, toluene, EtOH, $NaHCO_3$ satd., 14 h., quant.; **b)** BBr_3 (4.2 eq.), CH_2Cl_2, 0 °C to r.t., 3 h., 98%; **c)** 2-dimethyl aminoethyl chloride hydrochloride (2.2 eq.), Cs_2CO_3 (4.4 eq.), DMF, r.t. to 100 °C, 2 h., 65%; **d)** same procedure as **c)** with 3-dimethylaminopropyl chloride hydrochloride (2.2 eq.), 67%; **e)** 1-bromo-2-benzyloxyethane (2.2 eq.), Cs_2CO_3 (2.2 eq.), DMF, r.t. to 100 °C, 1 h., 63%; **f)** $ClCH_2CN$ (3.0 eq.), K_2CO_3 (2.2 eq.), acetone, 24 h, 70%; **g)** from **7**, BBr_3 (6.2 eq.), CH_2Cl_2, 0 °C to r.t., 2 h., 97%; from **8** : **h)** BH_3.THF (10.0 eq.), THF, rflx, 15 h, 50% **i)** ethylenediamine, P_2S_5 (cat.), 120 °C, 4 h, 80%.

2. Chemistry

In a recent article we reported the synthesis of various 2,5- and 2,6-diphenylpyridinyl derivatives [10]. A similar synthetic sequence was d eveloped to prepare the new 2,6-disubstituted pyrazine compounds (Scheme 1). Starting from 2,6-dichloropyrazine **1**, a Suzuki biphasic cross coupling procedure using the boronic acid **2** in excess (2.5 eq) afforded the bis derivative **3** in a quantitative yield [11–13]. The replacement of 2,6-dichloropyrazine **1** by the 2,6-dibromopyridine gave equivalent results with similar yields and times of reaction to obtain the bis-substituted compound **3**. The demethylation of **3** with BBr_3 at room temperature led to the phenolic compound **4** in an excellent 98% yield. A synthesis of compounds **3** and **4**, using Grignard derivatives in presence of dichloropyrazine and $Ni(dpp)_2Cl_2$ in THF at room temperature, has been previously reported in the literature [14]. In this case, the yield (only given for compound **4**) was limited to 55%.

The alkylation of **4** was performed using different halides and cesium carbonate as a base in DMF at 100 °C. The 2-chloroethyldimethylamine hydrochloride salt is first neutralized for 30 min at room temperature using cesium carbonate in DMF prior to add this solution to compound **4**. Compound **5** was obtained after 2 h in 65% yield. It is important to follow this two-steps procedure because we noted that the yield of this reaction dropped dramatically when all components were added altogether in one time. Modifying the nature of the base or the replacement of DMF by THF gave much lower yields. Similar conditions used for the 3-dimethylaminopropyl chloride hydrochloride salt afforded compound **6** in 57% yield. The other alkylation reactions did not require any pretreatment of the halogenated compound. Thus the 1-benzyloxy-2-bromoethane was reacted with **4** in the presence of cesium carbonate to give **7**. This reaction was completed after 2 h but the yield of **7** was relatively modest (63%). No starting material was recovered and there was also no detectable trace of the slight excess of 1-benzyloxy-2-bromoethane used. The last alkylation reaction was performed with chloroacetonitrile [15]. The optimization of this reaction leads us to use K_2CO_3 as a base in refluxing acetone for 24 h to afford compound **8** in 70% yield. In a general manner, one can imagine that the moderate yield of all the aforementioned phenol alkylation results from an unwanted reaction of the nitrogen pyrazine atoms providing highly water soluble compounds which were lost during the washes or make the purification more difficult by flash chromatography.

We have performed some reactions to generate the useful hydrophilic functions masked on compounds **7** and **8** (Scheme 1). Debenzylation was performed on **7** using BBr_3 at room temperature for 2 h to afford the bis alcohol **9** in 98% yield. Nitrile functions on **8** were first used to obtain primary amines and then to generate dihydroimidazole rings. In each case, we adapted the experimental conditions to obtain only symmetrical compounds. First reduction of the nitrile **8** was realized in the presence of a large excess of 1 M borane solution in refluxing THF for 15 h. Despite our numerous efforts, the compound **10** was obtained only in a 50% yield. Other reducing agents such as $LiAlH_4$ gave lower yields. Heating **8** in freshly distillated ethylenediamine with a catalytic amount of P_2S_5 led to the imidazoline compound **11** in 80% yield after only 4 h [16].

3. Results

3.1. DNA interaction

Fig. 1 illustrates the absorption spectra of compounds **5** and **11** in an aqueous buffer in the presence of increasing amounts of calf thymus DNA. In both cases, the binding of the drugs to DNA results in considerable spectral changes, characterized by a large bathochromic shift (16 nm) and a

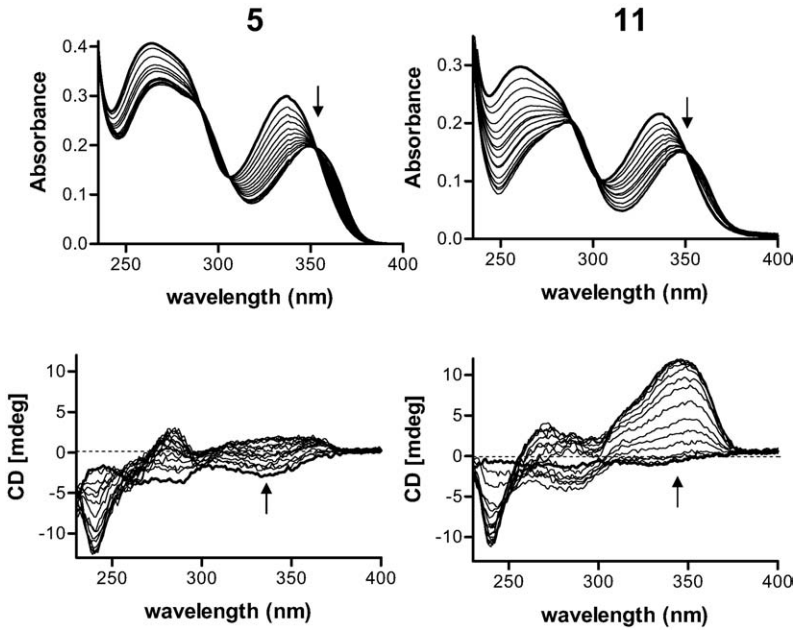


Fig. 1. (Top) Absorption and (bottom) CD DNA titration of compounds **5** and **11** in BPE buffer pH 7.1 (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA). Aliquots of a concentrated calf thymus DNA solution were added to 1 ml of a drug solution (20 μM for the absorbance and 50 μM for the CD measurements). The drug/DNA ratios increased from 0 to 20 (arrows).

marked hypochromism (about 30%). The red-shift of the drug absorption band was very pronounced with **5**, **6** and **11**, whereas there was no shift with **4** and **9** (Table 1). These two latter compounds bear OH terminal groups on both sides of the diphenylpyrazine core and are uncharged. The extent of binding is increased when the OH terminal groups of **9** are replaced with NH_2 groups in **10** and the substitution of these amines with methyl substituents, as in **5**, further promotes DNA interaction, as expected. The length of the alkyl chain with two or three CH_2 units makes no difference in terms of spectral changes and the melting temperature measurements also indicate that these two compounds **5** and **6** exhibit similar affinities for DNA. The Δ*T*m values ($Tm^{\text{drug–DNA complex}} - Tm^{\text{DNA alone}}$) reach about 16 °C calf thymus DNA (42% GC bp) at a drug/DNA ratio of 0.5 whereas a Δ*T*m of about 7 °C was recorded with the free amino compound **10** (Table 1). There was no increase of the *T*m in the presence of the uncharged OH compounds **4** and **9**, whatever the DNA used, be it with calf thymus DNA or the synthetic alternating homopolymer poly(dAT)$_2$. Fig. 2 illustrates the concentration-dependence for the thermal stabilization of poly(dAT)$_2$ by the different compounds. The magnitude of the melting temperature shifts measured with poly(dAT)$_2$ suggests that the affinity of the compounds for DNA rank in the order: **11** > **5**, **6** > **10** >>> **9**, **4**. The imidazoline compound shows stronger binding to DNA than the other compounds in the series. The Δ*T*m value reaches 30 °C at a **11**/poly(dAT)$_2$ ratio of 0.5. This corresponds to a reasonably high affinity for DNA. For this compound, an apparent binding constant of $K_{app} = 4.3$ (± 0.5) 10^5 M^{-1} was measured using an ethidium displacement fluorescence assay. This value is six times higher than that calculated for **10** ($K_{app} = 7.3$ (± 0.8) 10^4 M^{-1}) for example.

Table 1
DNA binding properties

	λ^{max}			Hypochromism %	Δ*T*m[a] (°C)
	Free (nm)	Bound (nm)	Δλ (nm)		
4	343	343	0	9.6	0
5	338	354	16	33.7	15.2
6	340	356	16	33.5	17.1
9	342	342	0	0.7	0
10	338	350	12	7.7	6.8
11	334	350	16	28.2	20.5

[a] *T*m measurements were performed in BPE buffer pH 7.1 (6 mM Na_2HPO_4, 2 mM $Na_2H_2PO_4$, 1 mM EDTA) using 10 μM drug and 20 μM calf thymus DNA (nucleotide concentration) with a heating rate of 1 °C min^{-1}.

The important spectral changes (large red-shift and hypochromism) observed with **11** or **5** (Fig. 1) in the presence of DNA can be attributed to the binding of the drugs in a lower

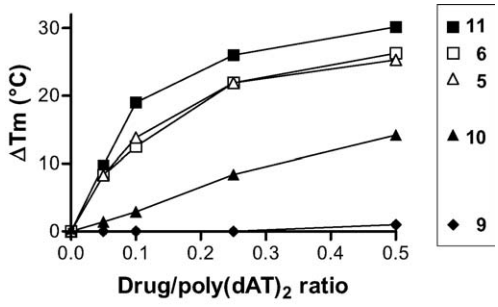


Fig. 2. Melting temperature variation Δ*T*m ($Tm^{\text{drug–DNA complex}} - Tm^{\text{DNA alone}}$, in °C) of poly(dAT)$_2$ after incubation with the diphenylpyrazines at increasing drug/DNA-phosphate. *T*m measurements were performed in BPE buffer pH 7.1 (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA), in 1 cm quartz cuvettes at 260 nm with a heating rate of 1 °C min^{-1}. The *T*m values were obtained from first-derivative plots.

polarity environment relative to the bulk aqueous solvent. But different binding modes can account for the spectral perturbation and it is never possible to distinguish the mode of interaction of a drug with DNA from absorption measurements alone. Intercalation and groove binding can lead to similar and important spectral variations. Therefore, to approach the drug binding mechanism, we used circular dichroism (CD) which is one of the spectroscopic methods suitable to determine drug binding mode. As shown in Fig. 1, the addition calf thymus DNA to a solution of **11** induces a marked increase of the CD signal centered at 350 nm. A high positive induced CD is typically observed with DNA minor groove binders. In contrast, intercalating agents generally give no induced CD or weakly negative signals in the drug absorption band [17]. A positive CD signal was only recorded with **11**; there was very little induced CD with **5** at 345 nm (Fig. 1) and the same weak signals with **6** (data not shown). The imidazoline compound **11** which exhibits the highest affinity for DNA is also the only potent minor groove binder in the series, probably because of the terminal imidazole groups which may prevent the rapid dissociation of the drug–DNA complexes. Considering this, it is therefore easy to explain the footprinting gel presented in Fig. 3 which shows most clearly that **11** is the only molecule in the series which blocks the cleavage of DNA by the endonuclease DNase I at-specific sites. Clear footprints develop at several sites along the sequence of the 265-bp DNA restriction fragment used in these experiments in the presence of **11** at 5, 10 or 20 μM, whereas there was absolutely no effect with the other molecules, even at the highest concentration tested (20 μM). For the dimethylamino compounds **5** and **6**, either their affinity for DNA is not sufficiently high, or most likely they form insufficiently stable drug–DNA minor groove complexes. The densitometric analysis of the gel presented in Fig. 4 reveals that the dihydroimidazole molecule preferentially recognizes AT-rich DNA sequences. The footprints coincides with the position of three strictly AT sites: 5′-ATTA, 5′-ATTAA and 5′-AAAA (Fig. 4).

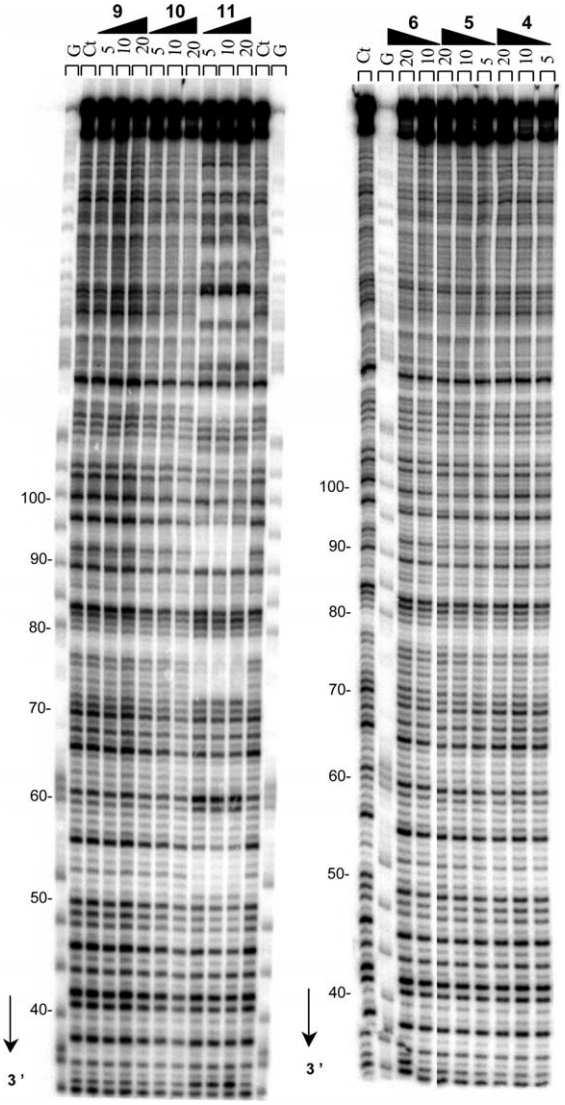


Fig. 3. DNase I footprinting of the diphenylpyrazines on the 265-bp *Eco*RI-*Pvu*II DNA fragment from pBS. The DNA was 3′-end labeled with [α-^{32}P]dATP in the presence of AMV reverse transcriptase. The products of nuclease digestion were resolved on an 8% polyacrylamide gel containing 8 M urea. Each drug was tested at 5, 10 or 20 μM. Control tracks (Ct) contained no drug. The tracks labeled "G" represent dimethylsulfate-piperidine markers specific for guanines.

3.2. Cytotoxicity

The antiproliferative activity of the molecules was investigated using human leukemia CEM cells after treatment for 72 h with the test drugs. IC_{50} values are collated in Table 2. As in the previous series of diphenylpyridine compounds [10], we regrettably found no direct correlation between DNA interaction and cytotoxicity for the pyrazine molecules. The potent DNA binder **11** shows no higher cytotoxicity than the two uncharged molecules **4** and **9** that practically do not bind to DNA. The most cytotoxic compounds are the dimethylaminoalkyl molecules **5** and **6**, with IC_{50} values in the ~5 μM range. An interesting point, however, is to compare the cytotoxicity of the pyrazine molecules with that of the correspond compounds **A**–**F** in the pyridine series (Table 2). In all cases, the pyrazines proved to be more cytotoxic that the pyridines. The two alcohol compounds **A** and **D** which were totally inactive in the pyridine series now become relatively cytotoxic in the pyrazine series. The same is true for the imidazoline compound for which the IC_{50} value is also significantly reduced as a consequence of the pyridine → pyrazine substitution. The additional heterocyclic nitrogen thus plays a role to promote the cytotoxic action of the molecules. This effect cannot be attributed to DNA recognition (the pyrazine and pyridine molecules show roughly comparable affinities for DNA) but it may be a consequence of a facilitated uptake in cells and/or interaction with targets other than nucleic acids. Some compounds in the series were found to inhibit two protein kinases (Dr. L. Meijer, CNRS, Roscoff, France, *personal communication*) and we cannot exclude the possibility that kinase inhibition contribute to the cytotoxic action in some cases.

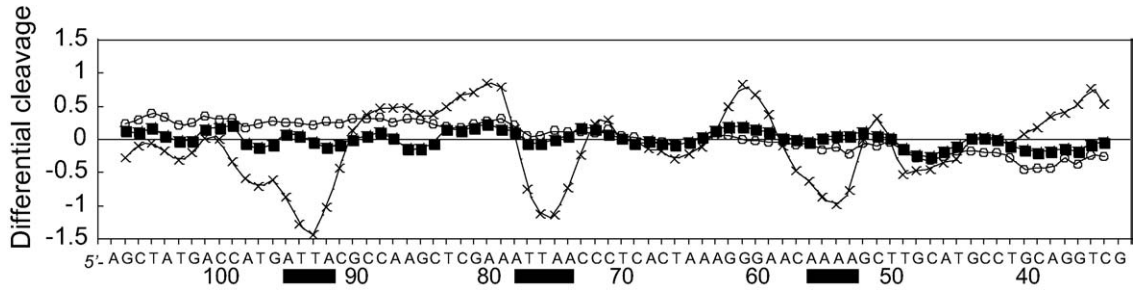
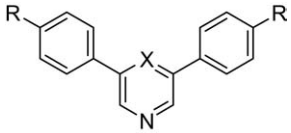


Fig. 4. Differential cleavage plots comparing the susceptibility of the 265-mer DNA fragment to DNase I cutting in the presence of 10 μM (×) **11**, (○) **9** and (■) **10**. Negative values correspond to a ligand-protected site and positive values represent enhanced cleavage. Vertical scales are in units of ln(fa) – ln(fc), where fa is the fractional cleavage at any bond in the presence of the drug and fc is the fractional cleavage of the same bond in the control, given closely similar extents of overall digestion. Each line drawn represents a three-bond running average of individual data points, calculated by averaging the value of ln(fa) – ln(fc) at any bond with those of its two nearest neighbors. Only the region of the restriction fragment analyzed by densitometry is shown.

Table 2
Cytotoxicity [IC_{50}][a]



	Pyridine (X = CH)		Pyrazine (X = N)	
R = OH	**A**	> 30	**4**	14 ± 1.9
R = O–$(CH_2)_2$–$N(CH_3)_2$	**B**	16.3 ± 1.4	**5**	5.7 ± 1.6
R = O–$(CH_2)_3$–$N(CH_3)_2$	**C**	5.4 ± 0.5	**6**	4.8 ± 1.8
R = O–$(CH_2)_2$–OH	**D**	> 70	**9**	16.3 ± 0.3
R = O–$(CH_2)_2$–NH_2	**E**	25.6 ± 2.3	**10**	12.7 ± 2.5
R = O–CH_2-imidazole	**F**	40.9 ± 1.2	**11**	15.5 ± 3.1

[a] Drug concentration (μM) that inhibits CEM leukemia cell growth by 50% after incubation in liquid medium for 72 h.

In conclusion, we have synthesized novel diphenylpyrazine molecules and investigated their DNA binding and cytotoxic properties. Three major points can be raised from this study: (1) One compound, bearing imidazoline terminal groups, acts as a potent AT-specific DNA minor groove recognition element. (2) There is no relationship between DNA interaction and cytotoxicity but the replacement of the pyridine by a pyrazine ring promotes the cytotoxicity of the molecules. (3) A versatile synthetic method has been elaborated to build the bis-phenol-pyrazine unfused aromatic core which can easily substituted to afford a repertoire of symmetrical molecules functionalized with various side chains.

4. Experimental section

4.1. Chemistry

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker DPX 250 instrument using $CDCl_3$ or DMSO-d_6. The chemical shifts are reported in ppm (δ scale) and all coupling constants (J) values are in hertz (Hz). The splitting patterns are designated as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and dd (doublet doublet). Melting points are uncorrected. IR absorption spectra were obtained on a Perkin Elmer PARAGON 1000PC and values were reported in cm^{-1}. MS spectra (Ion Spray) were performed on a Perkin Elmer Sciex PI 300. Monitoring of the reactions was performed using silica gel TLC plates (silica Merck 60 F254). Spots were visualized by UV light at 254 and 356 nm. Columns chromatography were performed using silica gel 60 (0.063–0.200 mm, Merck).

*4.1.1. 2,6-Bis-(4-methoxy-phenyl)-pyrazine (**3**) [12,13]*

A solution of 2,6-dichloropyrazine **1** (500 mg, 3.36 mmol) and 4-methoxyphenylboronic acid (1.28 g, 8.39 mmol) in a mixture of toluene (33 ml), ethanol (20 ml) and aqueous saturated $NaHCO_3$ solution (14 ml) was degassed by argon bubbling for 20 min. $Pd(PPh_3)_4$ (10% mol) was added and the mixture was immediately transferred in an pre-heated oil bath and refluxed for 14 h. After hydrolysis (50 ml), the aqueous layers were extracted with ethyl acetate (2 × 50 ml), washed with brine (50 ml) then dried over $MgSO_4$, and filtered. The solvents were removed under reduced pressure and the crude material was purified by flash chromatography (ether petroleum/ethyl acetate 7:3) to afford compound **3** as a white solid (900 mg, quant.). Rf (ether petroleum/ethyl acetate 7:3): 0.38; m.p. 137 °C [18]; IR (KBr, cm^{-1}) ν 1605, 1513, 1426, 1305, 1249, 1165, 1017, 829; ^{1}H-NMR ($CDCl_3$, 250 MHz): δ 3.85 (s, 6H), 7.01 (d, 4H, J = 11.4 Hz), 8.07 (d, 4H, J = 11.4 Hz), 8.81 (s, 2H); ^{13}C-NMR ($CDCl_3$, 62.5 MHz): δ 55.4 (2 × CH_3), 114.4 (4 × CH), 128.4 (4 × CH), 129.2 (2 × Cq), 138.5 (2 × CH), 151.0 (2 × Cq), 161.2 (2 × Cq); MS (IS): 293 $(M + 1)^+$; Anal. calcd for $C_{18}H_{16}N_2O_2$: C, 73.96; H, 5.52; N, 9.58. Found: C, 73.68; H, 5.67; N, 9.69.

*4.1.2. 2,6-Bis-(4-hydroxy-phenyl)-pyrazine (**4**) [12,13]*

To a solution of compound **3** (1 g, 3.73 mmol) in CH_2Cl_2 (20 ml) at 0 °C, BBr_3 (15.67 ml, 1 M in CH_2Cl_2, 15.67 mmol) was added dropwise. After 3 h at room temperature, the reaction mixture was poured into ice (100 g), the aqueous layers were neutralized with $NaHCO_3$ then successively extracted with ethyl acetate (2 × 50 ml), dried over $MgSO_4$, and filtered. The solvents were removed under reduced pressure and the crude material was purified by flash chromatography columns (dichloromethane/methanol 96:4) to afford compound **4** as a yellow solid (900 mg, 98%). Rf (dichloromethane/methanol 96:4): 0.25; m.p. 155 °C Dec.; IR (KBr, cm^{-1}) ν

3418, 2648, 1609, 1590, 1489, 1268, 1207, 1176, 833; ^{1}H-NMR (DMSO-d_6, 250 MHz) : δ 6.93 (d, 4H, J = 8.5 Hz); 8.10 (d, 4H, J = 8.5 Hz); 8.98 (s, 2H); ^{13}C-NMR (DMSO-d_6, 62.5 MHz): δ 115.8 (4 × CH), 126.8 (2 × Cq), 128.3 (4 × CH), 137.6 (2 × CH), 150.4 (2 × Cq), 159.3 (2 × Cq); MS (IS): 265 $(M + 1)^+$; Anal. calcd for $C_{16}H_{12}N_2O_2$: C, 72.72; H, 4.58; N, 10.60. Found: C, 73.05; H, 4.41; N, 10.73.

*4.1.3. 2,6-Bis-[4-(2-Dimethylamino-ethoxy)-phenyl]-pyrazine (**5**)*

To a solution of compound **4** (200 mg, 0.76 mmol) was added in one portion Cs_2CO_3 (543 mg, 1.67 mmol) in dry DMF (10 ml) under argon at room temperature. In parallel, a solution of 2-chloroethyldimethylamine hydrochloride (240 mg, 1.67 mmol) in DMF (10 ml) was stirred in presence of Cs_2CO_3 (741 mg, 2.27 mmol). After 30 min, this solution was added to the pyrazine solution. The mixture was heated up to 100 °C for 2 h. After 2 h, water (50 ml) was added and the aqueous layers were extracted successively with ethyl acetate (2 × 25 ml) and dichloromethane (2 × 25 ml). The combined organic layers were dried over $MgSO_4$, and filtered. The solvents were removed under reduced pressure and the crude material was purified by flash chromatography (dichloromethane/methanol/triethylamine 80:20:0.01) to afford compound **5** as a white solid (200 mg, 65%). Rf (dichloromethane/methanol/triethylamine 80:20:0.01): 0.43; m.p. 97 °C; IR (KBr, cm^{-1}) ν 2771, 1608, 1513, 1437, 1247, 1170, 1023, 833; ^{1}H-NMR ($CDCl_3$, 250 MHz): δ 2.37 (s, 12H), 2.78 (t, 4H, J = 5.6 Hz), 4.15 (t, 4H, J = 5.7 Hz), 7.06 (dd, 4H, J = 2.0, 6.9 Hz), 8.09 (dd, 4H, J = 1.8, 6.8 Hz), 8.84 (s, 2H); ^{13}C-NMR ($CDCl_3$, 62.5 MHz): δ 46.0 (4 × CH_3), 58.3 (2 × CH_2), 66.2 (2 × CH_2), 115.1 (4 × CH), 128.4 (4 × CH), 129.3 (2 × Cq), 138.5 (2 × CH), 151.1 (2 × Cq), 160.5 (2 × Cq); MS (IS): 407 $(M + 1)^+$; Anal. calcd for $C_{24}H_{30}N_4O_2$: C, 70.91; H, 7.44; N, 13.78. Found: C, 71.23; H, 7.30; N, 13.95.

*4.1.4. 2,6-Bis-[4-(3-Dimethylamino-propoxy)-phenyl]-pyrazine (**6**)*

Same procedure as described for **5**: Compound **4** (200 mg, 0.76 mmol), Cs_2CO_3 (543 mg, 1.67 mmol), DMF (10 ml), room temperature, 3-chloropropyldimethylamine hydrochloride (240 mg, 1.67 mmol), Cs_2CO_3 (741 mg, 2.27 mmol), 2 h, flash chromatography (dichloromethane/methanol/triethylamine 80:20:0.01). Compound **6** was obtained as a white solid (188 mg, 57%). Rf (dichloromethane/methanol/triethylamine 80:20:0.01): 0.53; m.p. 76 °C; IR (KBr, cm^{-1}) ν 2942, 2768, 1607, 1515, 1432, 1248, 1176, 1055, 828; ^{1}H-NMR ($CDCl_3$, 250 MHz) : δ 1.99 (q, 4H, J = 6.5 Hz), 2.27 (s, 12H), 2.48 (t, 4H, J = 6.9 Hz), 4.09 (t, 4H, J = 6.6 Hz), 7.03 (d, 4H, J = 8.7 Hz), 8.08 (d, 4H, J = 8.9 Hz), 8.82 (s, 2H); ^{13}C-NMR ($CDCl_3$, 62.5 MHz): δ 27.6 (2 × CH_2), 45.6 (4 × CH_3), 56.4 (2 × CH_2), 66.4 (2 × CH_2), 114.9 (4 × CH), 128.3 (4 × CH), 129.1 (2 × Cq), 138.5 (2 × CH), 151.1 (2 × Cq), 160.6 (2 × Cq); MS (IS): 435 $(M + 1)^+$; Anal. calcd for $C_{26}H_{34}N_4O_2$: C, 71.86; H, 7.89; N, 12.89. Found: C, 71.54; H, 7.70; N, 13.07.

*4.1.5. 2,6-Bis-[4-(2-benzyloxy-ethoxy)-phenyl]-pyrazine (**7**)*

A solution of **4** (500 mg, 1.89 mmol) and Cs_2CO_3 (1.36 g, 4.17 mmol) in DMF (30 ml) was stirred under argon at room temperature, for 30 min. 1-Bromo-2-benzyloxyethane (719 µl, 4.54 mmol) was added and the mixture was heated up to 100 °C. After 2 h, water (30 ml) was added and the aqueous layers were extracted with ethyl acetate (3 × 50 ml). The combined organic layers were washed with brine (3 × 150 ml), dried over $MgSO_4$, and filtered. The solvents were removed under reduced pressure and the crude material was purified by flash chromatography (ethyl acetate/ether petroleum 1:1) to afford compound **7** as a white solid (630 mg, 63%). Rf (ethyl acetate/ether petroleum 1:1): 0.39; m.p. 87 °C; IR (KBr, cm^{-1}) ν 2882, 1606, 1514, 1431, 1244, 1100, 831; ^{1}H-NMR ($CDCl_3$, 250 MHz): δ 3.85 (dd, 4H, J = 3.2, 5.0 Hz), 4.21 (t, 4H, J = 4.6 Hz), 4.64 (s, 4H), 7.05 (d, 4H, J = 8.7 Hz), 7.28–7.39 (m, 10H), 8.08 (dd, 4H, J = 1.8, 7.7 Hz), 8.82 (s, 2H); ^{13}C-NMR ($CDCl_3$, 62.5 MHz): δ 67.6 (2 × CH_2), 68.5 (2 × CH_2), 73.5 (2 × CH_2), 115.1 (4 × CH), 127.8 (5 × CH), 128.4 (4 × CH), 128.5 (5 × CH), 129.4 (2 × Cq), 138.0 (2 × Cq), 138.5 (2 × CH), 151.1 (2 × Cq), 160.4 (2 × Cq); MS (IS) : 533 $(M + 1)^+$; Anal. calcd for $C_{34}H_{32}N_2O_4$: C, 76.67; H, 6.06; N, 5.26. Found: C, 76.34; H, 6.21; N, 5.38.

*4.1.6. 2,6-Bis-(4-cyanomethoxy-phenyl)-pyrazine (**8**)*

To a solution of compound **4** (1.20 g, 4.54 mmol) in acetone (50 ml), K_2CO_3 (1.38 g, 10.00 mmol) and chloroacetonitrile (864 µl, 13.65 mmol) were added. After 24 h at reflux, the reaction mixture was cooled to room temperature, water was added (100 ml), and the mixture was extracted with EtOAc (3 × 50 ml). The organic layers were then dried over $MgSO_4$, and filtered. The solvents were removed under reduced pressure and the residue was crystallized in a mixture of ethyl acetate/petroleum ether 90:10, to afford compound **8** as a white solid (1.09 mg, 70%). Rf (petroleum ether/ethyl acetate 1:1): 0.13; m.p. 148 °C; IR (KBr, cm^{-1}) ν 2980, 2250, 1607, 1512, 1437, 1287, 1225, 1172, 831; ^{1}H-NMR ($CDCl_3$, 250 MHz): δ 4.86 (s, 4H), 7.14 (d, 4H, J = 8.7 Hz), 8.16 (d, 4H, J = 8.7 Hz), 8.91 (s, 2H); ^{13}C-NMR ($CDCl_3$, 62.5 MHz) : δ 53.7 (2 × CH_2), 115.0 (2 × Cq), 115.6 (4 × CH), 128.9 (4 × CH), 131.7 (2 × Cq), 139.4 (2 × CH), 150.2 (2 × Cq), 158.15 (2 × Cq); MS (IS): 343 $(M + 1)^+$; Anal. calcd for $C_{20}H_{14}N_4O_2$: C, 70.17; H, 4.12; N, 16.36. Found: C, 69.80; H, 4.26; N, 16.50.

*4.1.7. 2,6-Bis-[4-(2-hydroxy-ethoxy)-phenyl]-pyrazine (**9**)*

To a solution of compound **7** (531 mg, 1 mmol) in CH_2Cl_2 (20 ml) at 0 °C, BBr_3 (6.18 ml, 1 M in CH_2Cl_2, 6.18 mmol) was added dropwise. After 3 h at room temperature, the reaction mixture was poured into ice (100 g), extracted with EtOAc (2 × 30 ml) then dried over $MgSO_4$, and filtered. The solvents were removed under reduced pressure and the crude material was purified by flash chromatography (dichloromethane/methanol 90:10) to afford compound **9** as a yellow solid (350 mg, 98%). Rf (dichloromethane/methanol 90:10):

0.54; m.p. 191 °C Dec.; IR (KBr, cm^{-1}) ν 3380, 2926, 1607, 1514, 1433, 1253, 1177, 1052, 827; ^{1}H-NMR (DMSO-d_6, 250 MHz): δ 3.75 (t, 4H, J = 5.2 Hz), 4.08 (t, 4H, J = 4.7 Hz), 7.12 (d, 4H, J = 8.7 Hz), 8.21 (d, 4H, J = 9.3 Hz), 9.07 (s, 2H); ^{13}C-NMR (DMSO-d_6, 62.5 MHz): δ 59.5 (2 × CH_2), 69.7 (2 × CH_2), 114.9 (4 × CH), 128.3 (4 × CH), 129.2 (2 × Cq), 138.3 (2 × CH), 150.1 (2 × Cq), 160.3 (2 × Cq); MS (IS): 353 $(M + 1)^+$; Anal. calcd for $C_{20}H_{20}N_2O_4$: C, 68.17; H, 5.72; N, 7.95. Found: C, 67.86; H, 5.89; N, 8.08.

*4.1.8. 2,6-Bis-[4-(2-amino-ethoxy)-phenyl]-pyrazine (**10**)*

To a solution of compound **8** (200 mg, 0.58 mmol) in THF (15 ml) at room temperature, BH_3·THF (5.85 ml, 1 M in THF, 5.85 mmol) was added dropwise. After 15 h at reflux, the reaction mixture was cooled to 0 °C, and then methanol was added dropwise to neutralize the excess of BH_3·THF. The solvents were removed under reduced pressure and the crude material was purified by flash chromatography (dichloromethane/methanol/triethylamine 1:1:0.01) to afford compound **10** as a hygroscopic solid (102 mg, 50%). Rf (dichloromethane/methanol/triethylamine 1:1:0.01): 0.15; m.p. > 250 °C IR (KBr, cm^{-1}) ν 3418, 2934, 2866, 2360, 1606, 1516, 1244, 1168, 828; ^{1}H-NMR (DMSO-d_6, 250 MHz): δ 2.88 (t, 4H, J = 5.1 Hz), 4.02 (t, 4H, J = 5.2 Hz), 7.12 (d, 4H, J = 8.7 Hz), 8.21 (d, 4H, J = 8.5 Hz), 9.07 (s, 2H); ^{13}C-NMR (DMSO-d_6, 62.5 MHz) : δ 59.3 (2 × CH_2), 69.3 (2 × CH_2), 113.9 (4 × CH), 127.3 (4 × CH), 127.0 (2 × Cq), 137.1 (2 × CH), 149.1 (2 × Cq), 160.2 (2 × Cq); MS (IS): 351 $(M + 1)^+$; Anal. calcd for $C_{20}H_{22}N_4O_2$: C, 68.55; H, 6.33; N, 15.99. Found: C, 68.82; H, 6.17; N, 15.84.

*4.1.9. 2,6-Bis-[4-(4,5-dihydro-1H-imidazol-2-yl-methoxy)-phenyl]-pyrazine (**11**)*

A stirred mixture of compound **8** (200 mg, 0.58 mmol), ethylenediamine (10 ml) (freshly distilled on KOH) and P_2S_5 (5 mg, 0.02 mmol) was heated at 120 °C in an oil bath for 4 h. The reaction mixture was then cooled, and the solvent was removed under reduced pressure. The crude material was purified by flash chromatography (dichloromethane/methanol/triethylamine 1:1:0.01) to afford compound **11** as a yellow solid (200 mg, 80%). Rf (dichloromethane/methanol/triethylamine 1:1:0.01): 0.15; m.p. 159 °C; IR (KBr, cm^{-1}) ν 3266, 2936, 2866, 1622, 1516, 1244, 1176, 1062, 828; ^{1}H-NMR (CD_3OD, 250 MHz): δ 3.67 (s, 8H), 4.78 (s, 4H), 7.14 (d, 4H, J = 8.9 Hz), 8.16 (d, 4H, J = 8.9 Hz), 8.89 (s, 2H); ^{13}C-NMR (CD_3OD, 62.5 MHz): δ 49.2 (2 × CH_2), 64.1 (4 × CH_2), 115.1 (4 × CH), 128.2 (4 × CH), 128.9 (2 × Cq), 138.8 (2 × CH), 149.9 (2 × Cq), 159.6 (2 × Cq), 163.2 (2 × Cq); MS (IS): 429 $(M + 1)^+$; Anal. calcd for $C_{24}H_{24}N_6O_2$: C, 67.27; H, 5.65; N, 19.61. Found: C, 67.53; H, 5.48; N, 19.50.

4.2. Biological studies

4.2.1. Absorption spectroscopy and melting temperature studies

Absorption spectra and melting curves were measured using an Uvikon 943 spectrophotometer coupled to a Neslab RTE111 cryostat. The *T*m measurements were performed in BPE buffer pH 7.1 (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA). The temperature inside the cuvette (10 mm pathlength) was increased over the range 20–100 °C with a heating rate of 1 °C min^{-1}. The "melting" temperature *T*m was taken as the mid-point of the hyperchromic transition.

4.2.2. CD spectroscopy

CD spectra were recorded on a J-810 Jasco dichrograph. Solutions of drugs, DNA and their complexes (1 ml in BPE buffer) were scanned in 1 cm quartz cuvettes. Measurements were made by progressive addition of concentrated calf thymus DNA to a drug solution at 50 μM to obtain the desired drug/DNA ratio. Five scans were accumulated and automatically averaged.

4.2.3. Fluorescence measurements

An experimental procedure previously described was followed [19,20].

4.2.4. DNase I footprinting

The experimental procedure has been recently detailed [21].

4.2.5. Cell cultures and survival assay

CEM human leukemia cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 medium, supplemented with 10% fetal bovine serum, glutamine (2 mM), penicillin (100 UI ml^{-1}) and streptomycin (100 μg ml^{-1}). The cytotoxicity of the studied molecules was assessed using a cell proliferation assay developed by Promega (CellTiter 96® AQ_{ueous} one solution cell proliferation assay). Briefly, 2×10^4 exponentially growing cells were seeded in 96-well microculture plates with various drug concentrations in a volume of 100 μl. After 72 h incubation at 37 °C, 20 μl of the tetrazolium dye solution were added to each well and the samples were incubated for a further 2 h at 37 °C. Plates were analyzed on a Labsystems Multiskan MS (type 352) reader at 492 nm.

Acknowledgments

This work was supported by grants (to A.L. and S.R.) from the Ligue Nationale Française contre le Cancer (comité du Nord, comité du Centre), (to S.R.) the Fondation pour la Recherche Médicale, the Cancéropôle grand Ouest and (to U.J.) from the Conseil Régional du Centre and CNRS. N.D. is the recipient of a fellowship from the Ligue Nationale Contre le Cancer. We thanks Dr. L. Meijer and O. Lozach for the CDK evaluation (CNRS, Roscoff, France).

References

[1] S. Neidle, DNA minor-groove recognition by small molecules, Nat. Prod. Rep. 18 (2001) 291–309.

[2] L.H. Hurley, DNA and its associated processes as targets for cancer therapy, Nat. Rev. Cancer 2 (2002) 188–200.
[3] P.B. Dervan, Molecular recognition of DNA by small molecules, Bioorg. Med. Chem. 9 (2001) 2215–2235.
[4] P.B. Dervan, B.S. Edelson, Recognition of the DNA minor groove by pyrrole-imidazole polyamides, Curr. Opin. Struct. Biol. 13 (2003) 284–299.
[5] D.S. Pilch, Z. Xu, Q. Sun, E. La Voie, L.F. Liu, K.J. Breslauer, A terbenzimidazole that preferentially binds and conformationally alters structurally distinct DNA duplex domains: A potential mechanism for topoisomerase I poisoning, Proc. Natl. Acad. Sci. USA 94 (1997) 13565–13570.
[6] A. Seaton, C. Higgins, J. Mann, A. Baron, C. Bailly, S. Neidle, H. Van den Berg, Mechanistic and anti-proliferative studies of two novel biologically active bis-benzimidazoles, Eur. J. Cancer 39 (2003) 2548–2555.
[7] B.S.P. Reddy, S.M. Sondhi, J.W. Lown, Synthetic DNA minor groove-binding drugs, Pharmacol. Ther. 84 (1999) 1–111.
[8] J.M. Woynarowski, Targeting critical regions in genomic DNA with AT-specific anticancer drugs, Biochim. Biophys. Acta 1587 (2002) 300–308.
[9] R.R. Tidwell, D.W. Boykin, Dicationic DNA minor groove binders as antimicrobial agents, in: M. Demeunynck, C. Bailly, W.D. Wilson (Eds.), DNA and RNA Binders, Wiley-VCH, 2003, pp. 414–460.
[10] U. Jacquemard, S. Routier, N. Dias, A. Lansiaux, J.F. Goossens, C. Bailly, J.L. Mérour, Synthesis of 2,5- and 3,5-diphenylpyridine derivatives for DNA recognition and cytotoxicity, Eur. J. Med. Chem. (2005) (in press).
[11] N. Schultheiss, E. Bosch, Facile synthesis of diarylpyrazines using Suzuki coupling of dichloropyrazines with aryl boronic acids, Heterocycles 60 (2003) 1891–1897.
[12] J.R. Tagat, S.W. McCombie, B.E. Barton, J. Jackson, J. Shortall, Synthetic inhibitors of interleukin-6. II. 3,5-diaryl pyridines and meta-terphenyls, Bioorg. Med. Chem. Lett. 5 (1995) 2143–2146.
[13] M. Burton, C. De Tollenaere, J.-F. Cavalier, C. Marchand, F. Dussart, J. Marchand-Brynaert, J.-F. Rees, Synthesis, structure-activity relationship and in vitro evaluation of coelenterazine and coelenteramine derivatives as inhibitors of lipid peroxidation, Free Rad. Res. 37 (2003) 145–148.
[14] J.R. Tagat, S.W. Mc Combie, B.E. Barton, J. Jackson, J. Shortall, Synthetic Iinhibitors of interleukin-6 II: 3,5-diarylpyridines and meta-terphenyls, Bioorg. Med. Chem. Lett. 5 (1995) 2143–2146.
[15] C. Liljebris, S.D. Larsen, D. Ogg, B.J. Palazuk, J.E. Bleasdale, Investigation of potential bioisosteric replacements for the carboxyl groups of peptidomimetic inhibitors of protein tyrosine phosphatase 1B: identification of a tetrazole-containing inhibitor with cellular activity, J. Med. Chem. 45 (2002) 1785–1798.
[16] M. Anastassioudou, S. Danoun, L. Crane, G. Baziard-Mouysset, M. Payard, D.H. Caignard, M.C. Rettori, P. Renard, Synthesis and pharmacological evaluation of imidazoline sites I_1 and I_2 selective ligands, Bioorg, Med. Chem. 9 (2001) 585–592.
[17] W.D. Wilson, F.A. Tanious, D. Ding, A. Kumar, D.W. Boykin, P. Colson, C. Houssier, C. Bailly, Nucleic acid interactions of unfused aromatic cations: evaluation of proposed minor-groove, major-groove and intercalation binding modes, J. Am. Chem. Soc. 120 (1998) 10310–10321.
[18] F. Tutin, Synthesis in the epinephrine series II. The formation and properties of 2,5 and 2,6-disubstituted pyrazines and their conversion into ketones and iminoketones, J. Chem. Soc. 97 (1910) 2495–2524.
[19] B.C. Baguley, W.A. Denny, G.J. Atwell, B.F. Cain, Potential antitumor agent. 34. Quantitative relationships between DNA binding and molecular structure for 9-anilino-acridines substituted in the anilino ring, J. Med. Chem. 24 (1981) 170–177.
[20] V. Pavlov, P. Kong Thoo Lin, V. Rodilla, Cytotoxicity, DNA binding and localisation of novel bis-naphthalimidopropyl polyamine derivatives, Chem. Biol. Interact. 137 (2001) 15–24.
[21] C. Bailly, J. Kluza, C. Martin, T. Ellis, M.J. Waring, DNase I footprinting of small molecule binding sites on DNA, Methods Mol. Biol. 288 (2004) 319–342.