Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 1721–1737
Bioorganic & Medicinal Chemistry

Synthesis, characterization and antiproliferative studies of the enantiomers of *cis*-[(1,2-camphordiamine) dichloro]platinum(II) complexes

Ángel M. Montaña,[a,*] Francisco J. Bernal,[a] Julia Lorenzo,[d] Carlos Farnós,[a] Consuelo Batalla,[a] Maria J. Prieto,[b] Virtudes Moreno,[c] Francesc X. Avilés,[d] Juan M. Mesas[e] and María-Teresa Alegre[e]

[a]*Departamento de Química Orgánica, Universidad de Barcelona, Martí i Franquès 1-11, 08028 Barcelona, Spain*
[b]*Departamento de Microbiología, Universidad de Barcelona, Campus de Pedralbes, 08028 Barcelona, Spain*
[c]*Departamento de Química Inorgánica, Universidad de Barcelona, Martí i Franquès 1-11, 08028 Barcelona, Spain*
[d]*Instituto de Biotecnología y Biomedicina Vicent Villar Palasí, Universidad Autónoma de Barcelona, Bellaterra, 08193 Barcelona, Spain*
[e]*Escuela Politécnica Superior, Universidad de Santiago de Compostela, Campus de Lugo, 27002 Lugo, Spain*

Received 10 July 2007; revised 2 November 2007; accepted 7 November 2007
Available online 13 November 2007

Abstract—The platinum(II) complex *cis*-[(1*S*,2*R*,3*S*)-1,7,7-trimethylbicyclo[2.2.1]heptane-2,3-diamine]dichloroplatinum(II) (**1**) and its enantiomer (**2**) have been synthesized and physically and spectroscopically characterized. To obtain the enantiopure complexes the *chiral pool approach* was applied. The synthetic pathway has four steps, starting from (±)-diphenylethylenediamine (DPEDA) (**3**) and the natural products (1*S*)-camphorquinone or (1*R*)-camphorquinone to obtain enantiomers **1** and **2**, respectively. The interaction of the Pt(II) complexes with DNA was studied by several techniques: circular dichroism, electrophoresis on agarose gel and atomic force microscopy (AFM). These studies showed differences in the degree of interaction between both enantiomers and DNA (calf thymus DNA and plasmid pBR322 DNA). The cytotoxicity of enantiomers **1** and **2** against the HL-60 cell line was studied by in vitro tests of antiproliferative activity, incubating during both 24 h and 72 h. An important difference of activity was found between both enantiomers regarding the IC_{50} data at 24 h of incubation. Thus, complex **1** showed to be much more active than its enantiomer **2**.

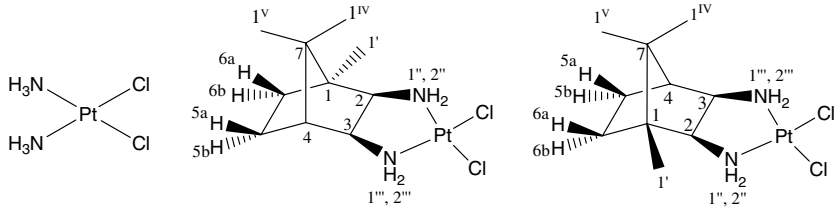
1. Introduction

The development of new platinum alkylating agents, different from the classical model of cisplatin[1,2] (Fig. 1), is actually an important research line in the search of less toxic, more potent and more selective chemotherapeutic anticancer agents. The high nephrotoxicity of cisplatin (partially avoided by using highly diluted saline solutions) and its high neurotoxicity (specially if administered together with paclitaxel) condition the therapeutic use of this active principle. It is used at low doses and in high dilution which conditions its efficiency.[3–6] Moreover many cancer types have been able to develop resistance against cisplatin[7,8] which considerably decreases its activity against cancer cells by several mechanisms, among them: (a) reduction of the drug concentration inside the cell by irreversible reaction with biomolecules containing nucleophilic sulfur groups, (b) reparation of the structural modifications undergone by DNA or (c) inactivation of the apoptotic mechanism of programmed cancer cell death.

In the last 10 years new alternative analogues of cisplatin have appeared such as carboplatin,[9–12] oxaliplatin[13–15] and other different types of platinum compounds (water soluble platinum compounds, multinuclear platinum complexes, compound with *trans* geometry, complexes having bioactive molecules (themselves) as ligands,

Keywords: 1,2-Camphordiamines; Enantiomers; Platinum(II) complexes; DNA; Atomic force microscopy; Circular dichroism; Anticancer agents; Cytotoxicity; Apoptosis.
* Corresponding author. Tel.: +34 93 402 16 81; fax: +34 93 339 78 78; e-mail: angel.montana@ub.edu

0968-0896/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2007.11.021



Cisplatin **1** **2**

Figure 1. Structure of cisplatin and of complexes **1** and **2**.

etc.),[16–19] in an effort to overcome the limitations of the actual platinum based therapeutic agents.[20]

There are in the literature references to structure–activity relationship studies, SAR, about the influence of stereochemistry in the activity of platinum(II) complexes. Thus, different antitumour activities have been found between diastereoisomers of ethylenediamino complexes, both in vitro and in vivo.[21] Also, a wide study on the influence of *cis* versus *trans* configuration on the cytotoxic activity of 1,2-diamino-cyclohexane platinum(II) complexes (DACH)[22] has been carried out, and very interesting differences were appreciated. Moreover, studies on the change of *erythro* into *threo* configuration in dihydroxylated DACH complexes showed a clear influence of stereochemistry in the molecular recognition of drugs by the pockets or receptors of target biomolecules, in this case, the bases of DNA.

In pharmacology, chirality is an important factor in drug efficacy. About 56% of the drugs currently in use are chiral compounds, and about 88% of these chiral synthetic drugs are used therapeutically as racemates. Unfortunately, there are many racemic drugs where the stereospecificity of the metabolism and/or the pharmacodynamic effects of the enantiomers are not known.[23]

Prior to Ariens' critical review of '*Sophisticated nonsense in pharmacokinetics and clinical pharmacology*',[24] published in 1984, neglect of stereochemistry in drug development was widespread and only in the last decade or so has it achieved a prominent place in druce the publication of this seminal work it is well established that there is a molecular recognition of enantiomers of chiral drugs by the chiral receptors in cells, so one of the enantiomers, the eutomer, is the active or more active one, but the other enantiomer, the distomer, is the inactive or less active one.

In the field of chiral platinum complexes, the interaction with DNA and formation of cross-links with adjacent purine bases are considered to be the crucial steps in the differentiation of antitumour activity between enantiomeric complexes. Because double-helical DNA has a chiral nature, interaction of DNA molecule with enantiomeric platinum complexes should lead to diastereoisomeric adducts. It has been demonstrated that DNA cross-links of platinum complexes with enantiomeric amine ligands not only can exhibit different conformational features but also can be processed differently by the cellular machinery as a consequence of these conformational and/or configurational differences.[25]

In the present work, we have synthesized, isolated and tested both enantiomers of camphor-1,2-diamine platinum(II) complexes (see Fig. 1) in order to evaluate the different cytotoxic activity of both enantiomers and to initiate the structure–activity relationship in this kind of compounds.

The synthesis of both enantiomeric camphane ligands was carried out in parallel and the corresponding platinum(II) complexes were prepared by reaction with potassium tetrachloroplatinate. Studies of interaction of these complexes with DNA were performed by three techniques: (a) Circular dichroism, that allowed us to study the changes produced on the secondary structure of DNA, by the bonding of platinum complexes to the DNA bases. (b) Electrophoresis on agarose gel, technique that allowed us to obtain information on the modification of the tertiary structure of DNA after interaction with platinum(II) complexes. (c) Atomic force microscopy (AFM), which showed, in an intuitive form, the topology and morphology of DNA when it interacts with platinum complexes. The evaluation of the cytotoxic activity of both enantiomers was studied in vitro against a HL-60 cancer cell line (leukaemia). Interesting differences were observed in the antiproliferative activity of both enantiomers and in the apoptotic death mechanism, as it is discussed below.

2. Results and discussion

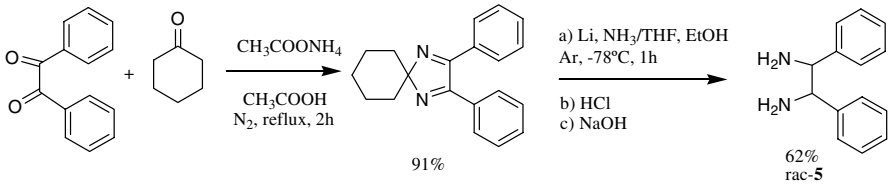
2.1. Synthesis and characterization of 1,2-diamine ligands and platinum complexes

The synthesis of ligands **3** and **4** was performed by the same synthetic pathway[26] (see Scheme 2). The starting material for **3** was (1*S*)-camphorquinone and (±)-1,2-diphenylethane-1,2-diamine (**5**) [(±)-DPEDA], meanwhile, **4** was prepared from (1*R*)-camphorquinone and (±)-DPEDA. A great advantage in the preparation of enantiomers **3** and **4** is the commercial availability of both enantiomers of the precursory camphorquinone. All intermediates and final products were physically and spectroscopically characterized by FT-IR, ^{1}H and ^{13}C NMR, as well as 2-D correlation experiments, and mass spectrometry and elemental analysis. Also, the optical activity was measured on enantiopure samples of products. The preparation of (±)-DPEDA (**5**) was

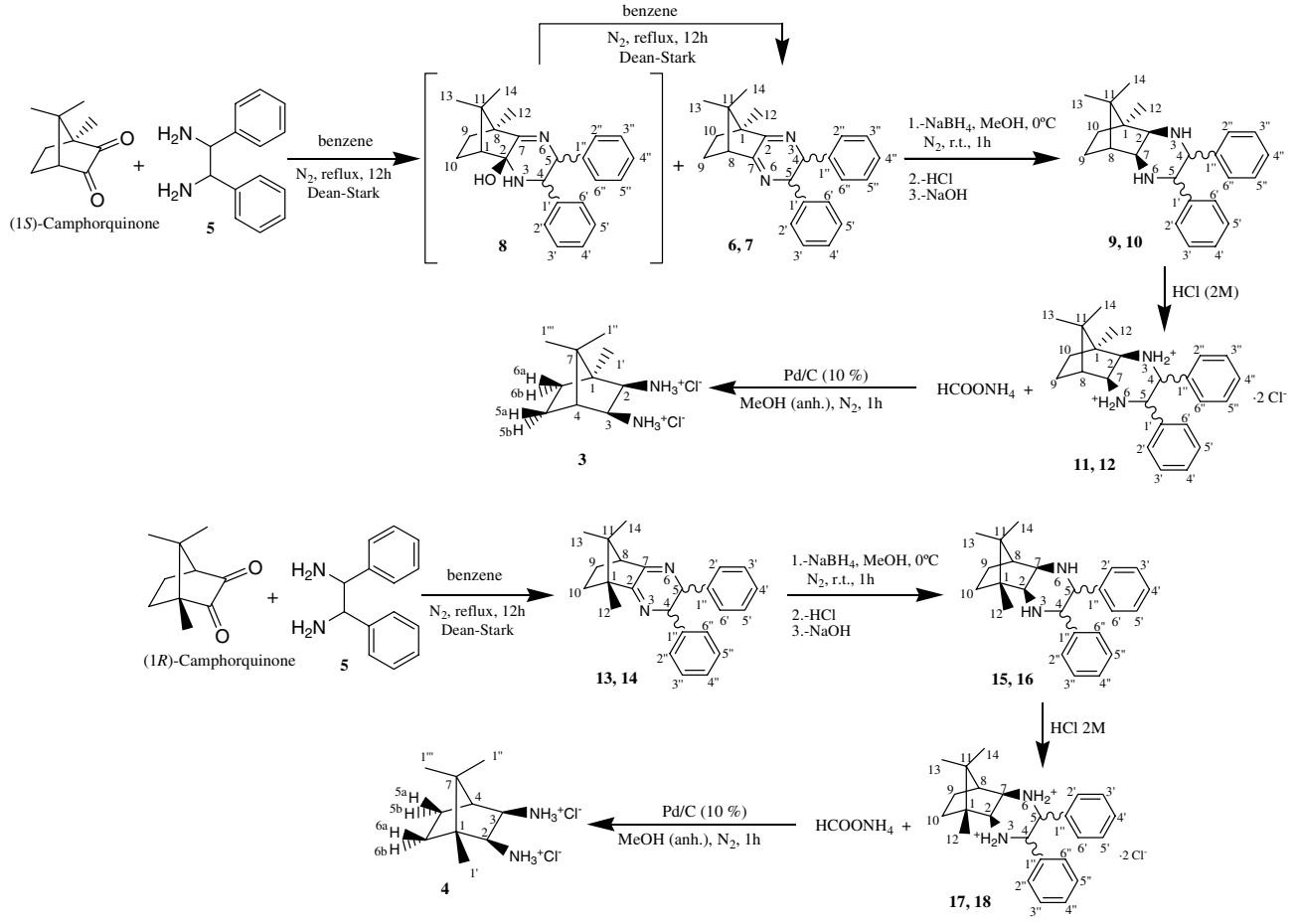
carried out in two steps (see Scheme 1) [27]: (1) Condensation reaction of dibenzoyl, cyclohexanone and ammonium acetate. (2) Reduction of the bisimine obtained from the previous reaction by the use of Li, NH_3/THF, EtOH at −78 °C, resulting in a racemic mixture of 1,2-diphenylethane-1,2-diamine (**5**).

The first step in the synthesis of camphordiamines is the condensation between camphorquinone and (±)-DPEDA (**5**) facilitated by the azeotropic distillation of water/toluene system in a Dean–Stark apparatus. The formation of diastereoisomers **6**, **7**, **13** and **14** (see Fig. 2) was due to the use of **5** as a racemic mixture. The byproduct **8**, isolated from the condensation reaction of (1*S*)-camphorquinone, is a reaction intermediate precursor of diimine **6**. This intermediate is a hemiaminal that was transformed into **6**, in quantitative yield, by a dehydration process under the same condensation reaction conditions.

The second step in the synthesis of ligands was the reduction of imino groups in **6**, **7**, **13** and **14** with $NaBH_4$. We found out that it was very important to work under mild reaction conditions and to establish a short reaction time to avoid the acid–base abstraction of benzylic hydrogens of **6**, **7**, **13** and **14** by hydride ions. Otherwise, the bisimine system of these substrates should afford pyrazine systems instead of undergoing a reduction process to generate piperazines **9**, **10** or **15**, **16**. For this reason, in order to get higher chemoselectivity, it was preferred to run the reactions for short reaction times, even though, the conversion was not completed and to recover the unconverted starting material which could be submitted again to the reduction conditions in a subsequent different process. In the piperazines the secondary amine groups adopt a *cis* relationship with respect to the camphane framework because in the reduction process the hydride ions can



Scheme 1. Synthetic methodology for the preparation of diamine **5**.



Scheme 2. Synthetic path for the preparation of ligands **3** and **4**.

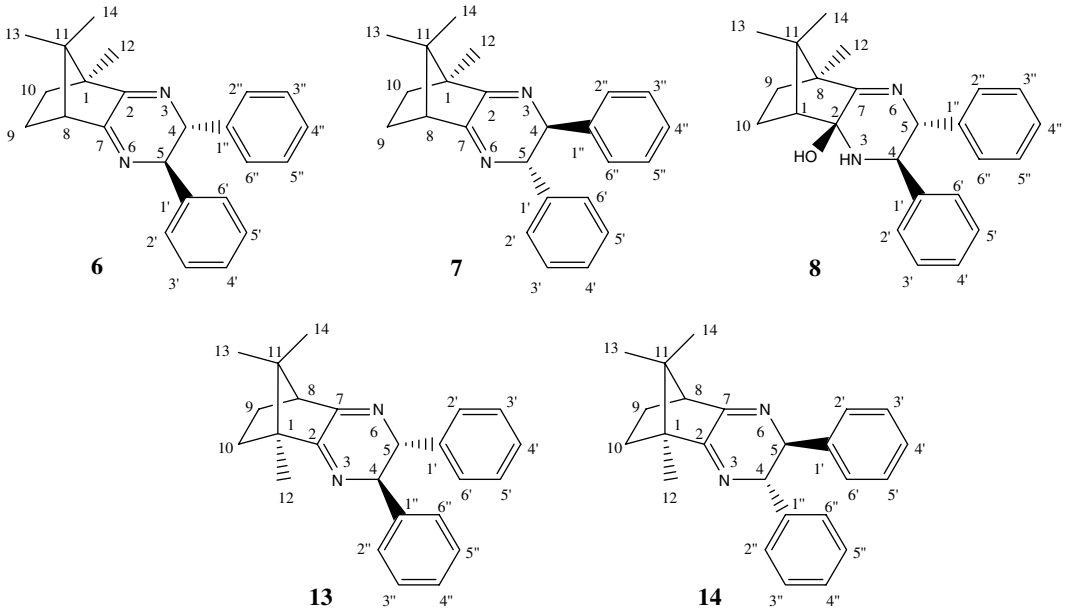
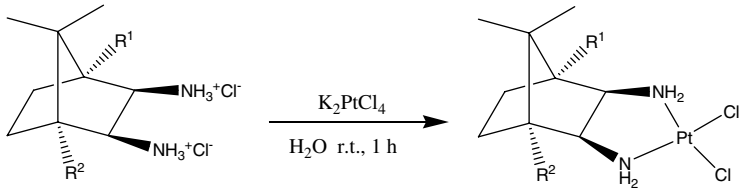


Figure 2. Structure of diastereoisomeric pairs (**6**, **7**) and (**13**, **14**) and of byproduct **8**.



3, R^1 = Me, R^2 = H
4, R^1 = H, R^2 = Me

1, R^1 = Me, R^2 = H
2, R^1 = H, R^2 = Me

Scheme 3. Preparation of Pt(II) complexes of 1,2-diamines **3** and **4**.

only attack the imino groups of **6**, **7**, **13** and **14** by the *endo* face of the bicyclic system.

The next step in the obtention of ligands **3** and **4** was the transformation of piperazines **9**, **10**, **15** and **16** into the hydrochlorides **11**, **12**, **17** and **18** by using 2 M HCl and ulterior concentration to dryness. Finally, the debenzylation process of these hydrochlorides was carried out by hydrogenolysis using 10% Pd/C as a catalyst and ammonium formate ($HCOONH_4$) as a source of hydrogen. In this reaction it is quite important the complete absence of O_2 (not only from the system atmosphere but also the possible oxygen dissolved in the absolute methanol used as solvent) in order to avoid the passivation of catalyst. The resulting products were the 1,2-diamines **3** or **4** as ammonium hydrochlorides, whose purification was performed by crystallization in MeOH.

The synthesis of platinum(II) complexes **1** and/or **2** was carried out by reaction of **3** and/or **4** with potassium tetrachloroplatinate in aqueous media, under nitrogen atmosphere and in the absence of light (see Scheme 3).

Complexes **1** and/or **2** precipitated once they were formed due to their low solubility in water, and they were isolated by filtration.

2.2. Studies of interaction of enantiomeric platinum complexes with DNA

With the purpose of performing a preliminary evaluation of the ability of complexes **1** and **2** to interact with DNA molecules as the main biological targets, several biochemical assays were recorded by using three techniques: circular dichroism, electrophoresis on agarose gel and atomic force microscopy. These techniques allowed us to observe the interaction of platinum complexes and DNA molecules at different levels.

2.3. Circular dichroism (CD) studies

This technique is quite sensitive to the changes in the secondary structure of nucleic acids, which allows analyzing any conformational modification of DNA provoked by its interaction with platinum complexes. When the complexes produce variations in the molar ellipticity $\Delta\theta$ this means that some modifications are also produced on the bases stacking and on the magnitude of the winding angle between adjacent base pairs, that is, on the bending and winding of DNA helix.[28,29]

In these CD studies, the spectra of compounds **1**, **2**, **3**, **4** and cisplatin were registered, after incubation with *Calf Thymus* DNA at three molar ratios r_i (molar ratio of the

compound per nucleotide): 0.1, 0.3 and 0.5. The wavelengths used were in between 220 and 320 nm. The values obtained of molar ellipticity for cisplatin and for the ligands **3**, **4** and complexes **1** and **2** are illustrated in Figure 3 and data are quoted in Table 1.

The ligands **3** and **4** did not provoke significant changes on the ellipticity (see Fig. 4A), however, the complexes **1** and **2** modified the bases stacking in DNA molecules, but to a lesser extent than cisplatin did. In the graphical representation of the net variation of maximum elliptic-

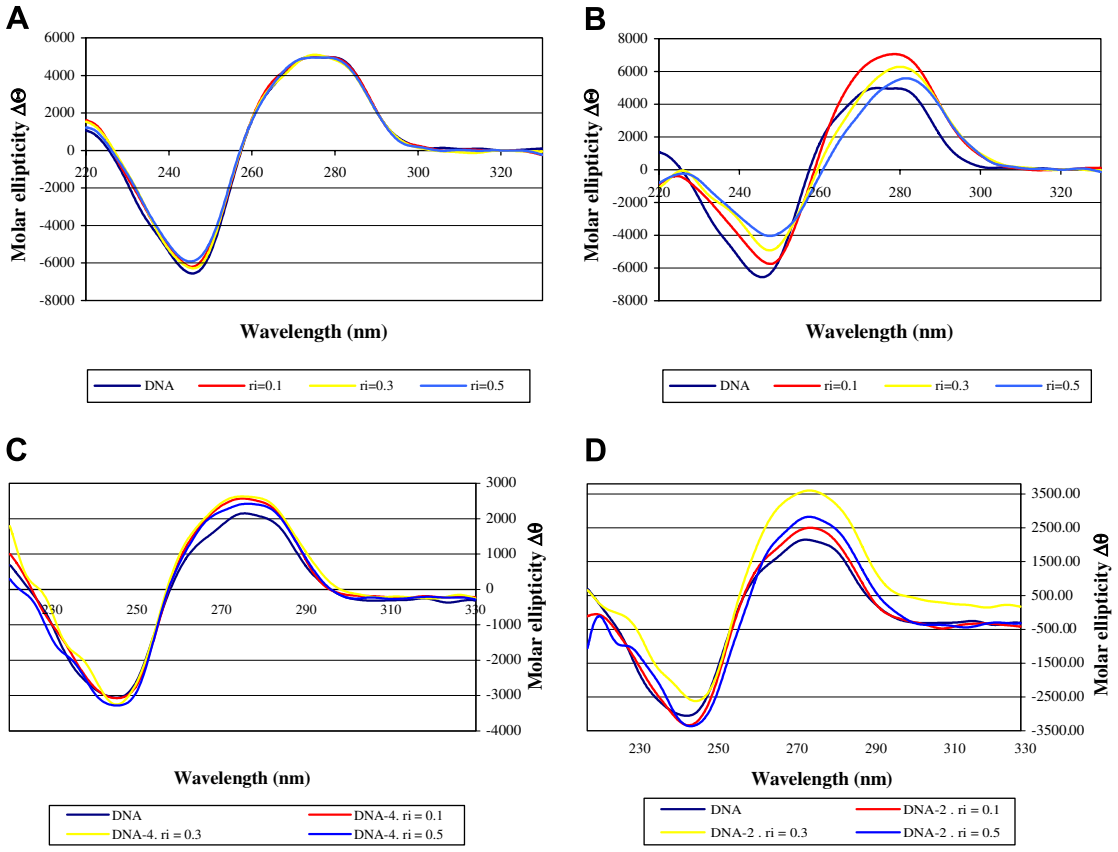


Figure 3. (A) CD curves of native DNA and DNA incubated with diamine **3** at $r_i = 0.1$, 0.3 and 0.5. (B) CD curves of native DNA and DNA incubated with platinum complex **1** at $r_i = 0.1$, 0.3 and 0.5. (C) CD curves of native DNA and DNA incubated with diamine **4** at $r_i = 0.1$, 0.3 and 0.5. (D) CD curves of native DNA and DNA incubated with platinum complex **2** at $r_i = 0.1$, 0.3 and 0.5.

Table 1. Ellipticity variations $\Delta\Theta$ and maximum and minimum wavelengths λ observed for **1**, **2**, **3**, **4** and cisplatin

Compound	r_i	$\Delta\Theta_{max}$	λ_{max}	$\Delta\Theta_{min}$	λ_{min}	$\Delta\theta_{max}$ (compound-DNA) − $\Delta\theta_{max}$ (free DNA)	λ_{max} (compound-DNA) − λ_{max} (free DNA)
DNA		4985	274.4	−6556	246.6		
	0.1	5006	275.6	−6203	245.6	21	1.2
Diamine **3**	0.3	5097	275.2	−6277	246.0	112	0.8
	0.5	4964	277.0	−5920	245.2	−21	2.6
	0.1	7060	278.6	−5746	247.8	2075	4.2
Complex **1**	0.3	6284	280.0	−4926	247.6	1299	5.6
	0.5	5587	281.6	−4042	247.6	602	7.2
	0.1	5405	274.4	−6546	246.6	420	0
Diamine **4**	0.3	5461	274.4	−6368	246.6	476	0
	0.5	5253	275.6	−6332	246.6	268	1.2
	0.1	7057	276.6	−5633	246	2072	2.2
Complex **2**	0.3	6435	276.2	−4882	247	1450	1.8
	0.5	5665	276.2	−5633	246	680	1.8
	0.1	7373	276.6	−5352	247.0	2388	2.2
Cisplatin	0.3	8355	279.6	−5158	248.4	3370	5.2
	0.5	7942	278.0	−4473	248.2	2957	3.6

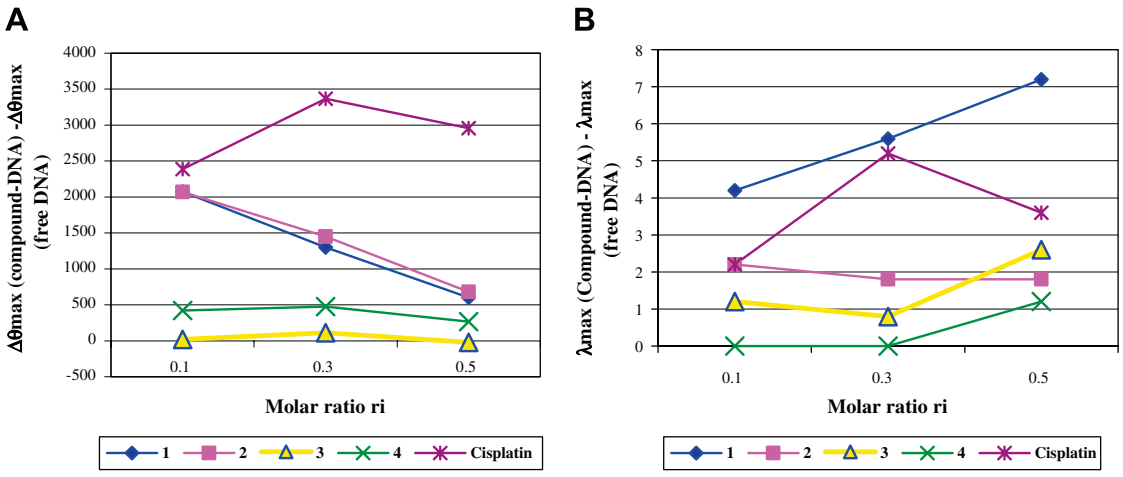


Figure 4. (A) Variations of maximum ellipticity $\Delta\theta$ of diamines **3** and **4**, complexes **1** and **2** and cisplatin versus native DNA. (B) Variations of maximum wavelength $\Delta\lambda_{max}$ of diamines **3** and **4**, complexes **1** and **2** and cisplatin versus native DNA.

ity versus r_i (Fig. 4A and B) it is possible to appreciate the different mode of interaction of enantiomers **1** and **2** with DNA.

When observing the ellipticity variation $\Delta\theta$ it is possible to appreciate that complexes **1** and **2** have behaviour quite similar to each other and quite different from that of cisplatin. When increasing the molar ratio r_i from 0.1 to 0.3 a progressive decrease of the ellipticity values is observed for **1** and **2** and an increase of ellipticity can be observed for cisplatin. This different behaviour could be interpreted by the easiness of cisplatin to form intra-strand *cis*-bifunctional bonds between the platinum atom and DNA due to steric reasons. When increasing the molar ratio from 0.3 to 0.5 the trend of the three complexes is similar (even maintaining the differences) and a decrease of molar ellipticity is observed in all cases. This fact could be related to the unwinding and bending of the DNA helix due to both, the formation of inter-strand bonds between DNA threads and to a possible change of the DNA B-form into a Z-form.

When analyzing the variations of wavelength at the maximum absorption, the trends are different for complexes **1** and **2** and the standard of cisplatin. In this case, complex **1** and cisplatin produce an increase of the wavelength when increasing the molar ratio from 0.1 to 0.3, meanwhile complex **2** induces a slight decrease of λ. The bathochromic effect observed for **1** could be interpreted as a higher possibility of **1** to form intra-strand *cis*-bifunctional bonds between platinum atom and DNA than complex **2**.[30] However, when going from 0.3 to 0.5 molar ratio the behaviour of both complexes and that of cisplatin are completely different. This phenomenon could be interpreted, as mentioned before, as due to the different degree of unwinding and bending of the DNA helix by the three compounds due to both, the formation of inter-strand bonds between DNA threads and to a possible change of the DNA B-form into a Z-form.

It is possible to conclude that the behaviour of the three complexes is highly dependent on the molar ratio r_i. From this study we can withdraw the conclusion that complexes **1** and **2** could have a slight different behaviour comparing maximum wavelength differences (at high molar ratios), which could be consistent with a possible different interaction mode for both enantiomeric platinum complexes with the chiral DNA target.

2.4. Agarose gel electrophoresis

The studies of electrophoresis on the samples of DNA incubated with the enantiomeric platinum complexes **1** and **2** allowed us to observe modifications on the tertiary structure of DNA molecules induced by the platinum complexes. In this study, plasmid pBR322 DNA was used, which presents a circular shape with to main forms: a relaxed open circular form (OC) and a supercoiled covalently closed form (CCC) that is much more compacted than the former one. *Cis*-platinum complexes interact with this type of DNA and produce a relaxation of the CCC form (causing a decrease of compaction) and, at the same time, they produce a certain folding of the OC form.[31–33] This phenomenon produces that the migration difference between the bands corresponding to both DNA forms should decrease when increasing the concentration of complexes. At sufficiently high drug concentrations, the coalescence point is reached in which both bands get superposed[34] at a medium position of the electrophoretic lane (see Fig. 5).

The 1,2-camphordiamine ligands **3** and **4** did not produce any change in the electrophoretic mobility of the plasmid DNA, which confirms the results obtained from the circular dichroism: the ligands do not interact with DNA. However, the enantiomeric complexes **1** and **2** clearly produced a change in the mobility of OC and CCC bands, reaching the coalescence point at both evaluated r_i. The only difference in the electrophoretic behaviour of both complexes **1** and **2** between $r_i = 0.1$ and $r_i = 0.5$ is that at the lower concentration both complexes seem to induce a less intense folding in the OC form, which would justify that their migration should be lower and, in consequence, the coalescence point should be slightly in an upper position with respect to the coalescence point induced at $r_i = 0.5$.

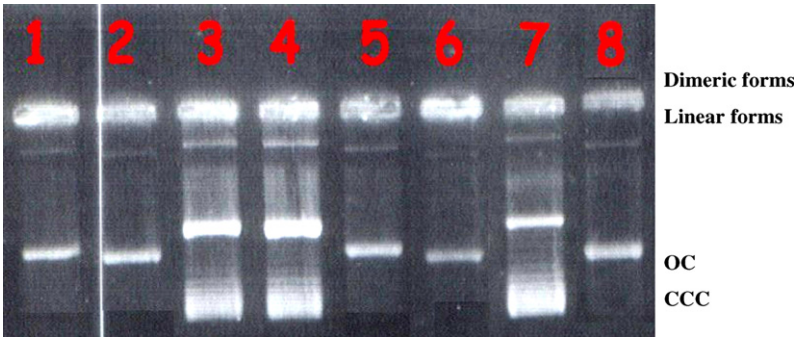


Figure 5. Agarose gel electrophoresis. Lane 1: pBR322 incubated with **1** at $r_i = 0.1$. Lane 2: pBR322 incubated with **1** at $r_i = 0.5$. Lane 3: pBR322 + **3** at $r_i = 0.5$. Lane 4: pBR322 + **4** at $r_i = 0.5$. Lane 5: pBR322 + **2** at $r_i = 0.1$. Lane 6: pBR322 + **2** at $r_i = 0.5$. Lane 7: free native pBR322. Lane 8: pBR322 incubated with cisplatin at $r_i = 0.5$.

2.5. Tapping-mode atomic force microscopy (TMAFM)

The atomic force microscopy resulted to be an excellent tool to study the surface relief, morphology and topology of the DNA molecules, before and after incubation with the compounds whose interaction with DNA was evaluated. With this technique it is possible to obtain three-dimensional pictures of DNA, which makes the observations very informative and intuitive. In our case the Tapping Mode AFM was used in order to minimize the contact between the tip of the probe and the surface to be analyzed. With this technique, the spoiling of the sample by contact and the risk of dragging and sweeping sample particles during the analyses were avoided.[35]

The evaluation of the degree of interaction of complexes **1** and **2** with DNA by TMAFM was carried out at a molar ratio $r_i = 0.005$. Also, diamine **3**, cisplatin (as standard or reference) and free plasmid DNA were studied by this technique. In the case of the free DNA test, the sample was heated up to 60 °C for 5 min to induce the adoption of the OC form by the DNA molecules. In all cases the same r_i concentration was used to make the test results comparable. The 2D photographic pictures of the AFM observations are illustrated in Figure 6.

The ligand **3** produced a higher aggregation degree than the free plasmid DNA but without inducing the formation of as many nodes and cross-links as the corresponding platinum complex **1**. The morphological singularities generated by every tested compound on the plasmid DNA are quoted in Table 2. The data from Table 2 represent the number of morphological changes undergone by DNA per 100 molecules evaluated. This number of molecules was taken into account for statistical reasons.

According to the previous studies of DC and electrophoresis, the ligand **3** does not interact with DNA. Thus, the morphological changes observed for this compound in AFM could be due to weak interactions like hydrogen bonds between the amines and the DNA bases. No covalent bonds are expected due to the low number of knots or cross-links observed. On the other hand, complexes **1** and **2** induced morphology changes on DNA molecules quite similar to that generated by cisplatin. However, complex **2** produces a higher degree of fragmentation of DNA chains and a lower number of knots than complex **1**. There are also some differences in the other morphological effects observed. This behaviour seems to indicate that the type and degree of interaction with DNA of both enantiomeric platinum complexes is slightly different.

2.6. Studies of the cytotoxicity in vitro of platinum complexes against HL-60 cells

Antiproliferative studies of enantiomers **1** and **2** were carried out with HL-60 cell line (acute promyelocytic leukaemia).[36,37] The cell viability was measured after incubation of cells in the presence of compounds to be tested at 37 °C for 24 and 72 h. In Figure 7 the viability (in %) versus concentration (μM) of complex **1** or **2** is represented. The IC_{50} values obtained for both enantiomers and cisplatin (as a standard or reference) after 24 and 72 h of incubation are quoted in Table 3.

Complex **1** is about twice as active as enantiomer **2**, at short incubation time. As a matter of fact, for a 100 μM concentration of **1**, after 24 h of incubation, only 10% of cells remain alive and functional. Meanwhile, for the same concentration of complex **2** and for the same incubation time 40% of cells are still alive. However, at higher incubation times both complexes improve their cytotoxic activities and reach similar IC_{50} values.

The explanation of this difference of activity between both enantiomers only could be based on their difference of chirality because all studies and tests have been carried out under strictly the same experimental conditions. The level at which this difference is produced is not known yet. It could be at the level of absorption of drug by the cell due to a higher ability of enantiomer **1** to pass through the cell membrane. Also, a possibility is that inside the cells enantiomer **2** should have more rapid deactivation kinetics in front of metallothioneins, glutathione and other biomolecules having nucleophilic thio groups. A simpler and alternative explanation is that enantiomer **1** could act as an eutomer inducing a better molecular recognition at the interaction sites of DNA bases inside the cell nucleus.

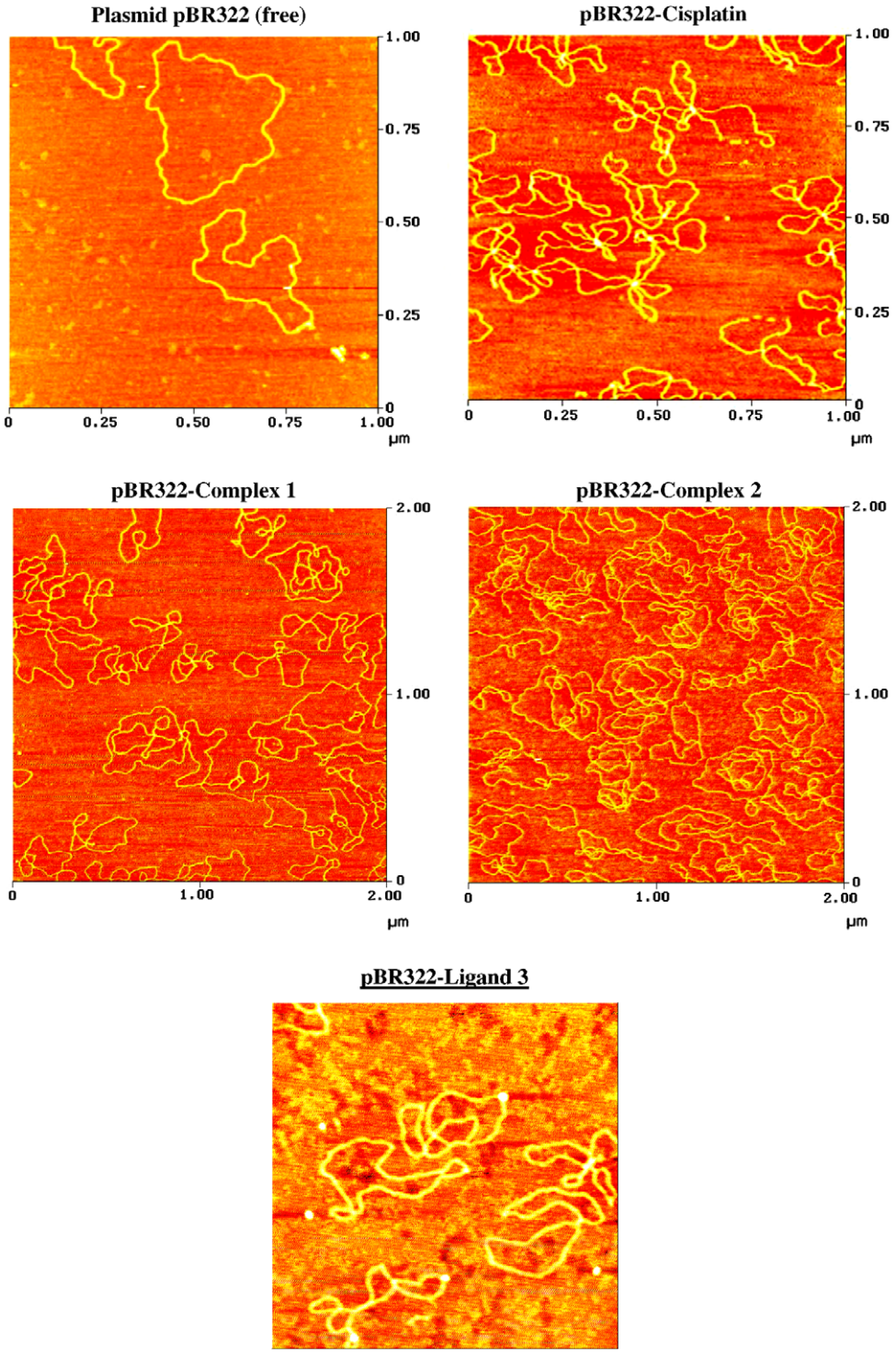


Figure 6. TMAFM images of plasmid DNA, free and incubated with **1**, **2**, **3** and cisplatin.

Table 2. Effects produced on pBR322 DNA, observed by AFM, after incubation with complexes **1**, **2** and cisplatin

Morphological effects	Free DNA	Ligand **3**	Complex **1**	Complex **2**	Cisplatin
Number of molecules of DNA evaluated	100	100	100	100	100
Number of fragments	0	8	10	51	18
Number of knots	3	19	186	123	212
Number of crossings	4	27	72	53	35
Number of supercoilings	0	22	55	35	47
Number of foldings	30	89	256	281	312

Ligand **3** was incubated with DNA and analyzed by AFM as a reference or standard for comparison. The data represent percentages.

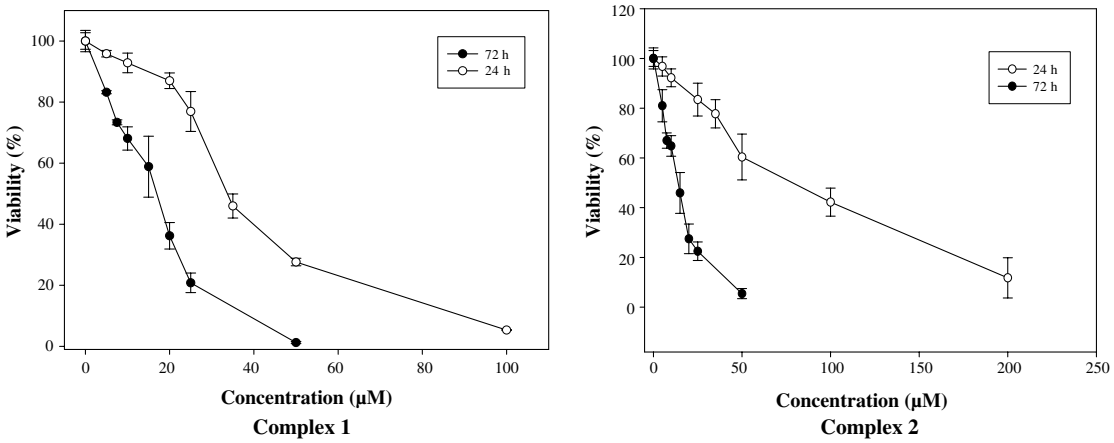


Figure 7. Viability of HL-60 cells in the presence of enantiomeric platinum complexes **1** and **2** after 24 and 72 h of incubation.

Table 3. IC_{50} data after 24 h and 72 h of incubation

Complex	IC_{50} (μM) a 24 h	IC_{50} (μM) a 72 h
1	35.25 ± 2.68	14.57 ± 2.38
2	71.48 ± 9.21	12.54 ± 1.34
Cisplatin	15.61 ± 1.15	2.15 ± 1.01

Complex **1** has a very different activity respect to its enantiomer **2** after 24 h of incubation.

2.7. Studies of apoptosis by flow cytometry

In order to study in which way the platinum compounds produced the cellular death (necrosis or apoptosis) studies of flow cytometry[38–44] were performed on enantiomers **1**, **2** and cisplatin as a reference. These compounds were incubated for 24 h at a concentration close to the IC_{50} and results are shown in Table 4. In Figure 8 the diagrams obtained by this technique, corresponding to the compounds **1**, **2** and cisplatin, as well as the control test, are illustrated. In these graphics, it is possible to appreciate the populations of cells in the four possible status: the population of alive cells (i.e., cells that present negative controls for annexin and propidium iodide PI (left square at the bottom), the population of cells that undergo apoptosis (negative for PI and positive for annexin, right square at the bottom), cells involved in a process of late apoptosis or necrosis (positive for both dyes, right square on the top) and necrotic cells (positive for PI, left square on the top).

From Figure 8 it is possible to observe the different behaviour of compounds **1** and **2**. Thus, **1** produces a higher population of apoptotic cells than **2**, even higher than cisplatin. This is the desirable death mechanism for a cytotoxic drug.

These assays showed that the studied compounds, in general, induced apoptosis and produced the death of the larger amount of cells by this pathway. In general low percentages of cell death by necrosis were observed. It is worth noting, as can be appreciated in Table 4 and Figure 8, the different behaviour of enantiomers **1** and **2** in the flow cytometry studies.

3. Conclusions

The work presented deals with the synthesis, evaluation of the interaction with DNA and the cytotoxic activity of two enantiomers of a new class of platinum(II) complexes with antiproliferative activity. These enantiomers showed interesting differences in their biological behaviour which demonstrate the molecular recognition of chirality by DNA as the target biomolecule.

We have carried out the enantioselective synthesis of ligands **3** and **4** with structure of 1,7,7-trimethylbicyclo[2.2.1]heptane-2,3-diamine. The enantiomeric excess obtained in both syntheses was of 90% for an overall yield of 30%. The preparation of enantiomeric platinum(II) complexes **1** and **2** was performed by reaction with K_2PtCl_4, affording pure samples of enantiomeric complexes which were purified and physically and spectroscopically characterized.

The interaction of both enantiomers with DNA was studied by three techniques: circular dichroism (to evaluate modifications on the secondary structure), electrophoresis on agarose gel (to estimate the modifications of the tertiary structure of DNA) and atomic force microscopy to observe the changes in morphology and topology of DNA. In the first assay we used Calf Thymus DNA and in the other two techniques pBR322 plasmid DNA.

Table 4. Percentages of the cell death pathways observed by the flow cytometry assay

Treatment (IC_{50} 24 h, μM)	% Vital ells	% Apoptotic ells	% Late apoptotic/necrotic cells	% Necrotic cells
Control	85.39	8.43	5.95	0.24
Cisplatin (15.61)	53.89	40.68	4.89	0.55
2 (78.41)	64.59	29.21	5.86	0.34
1 (35.25)	44.75	47.23	7.13	0.90

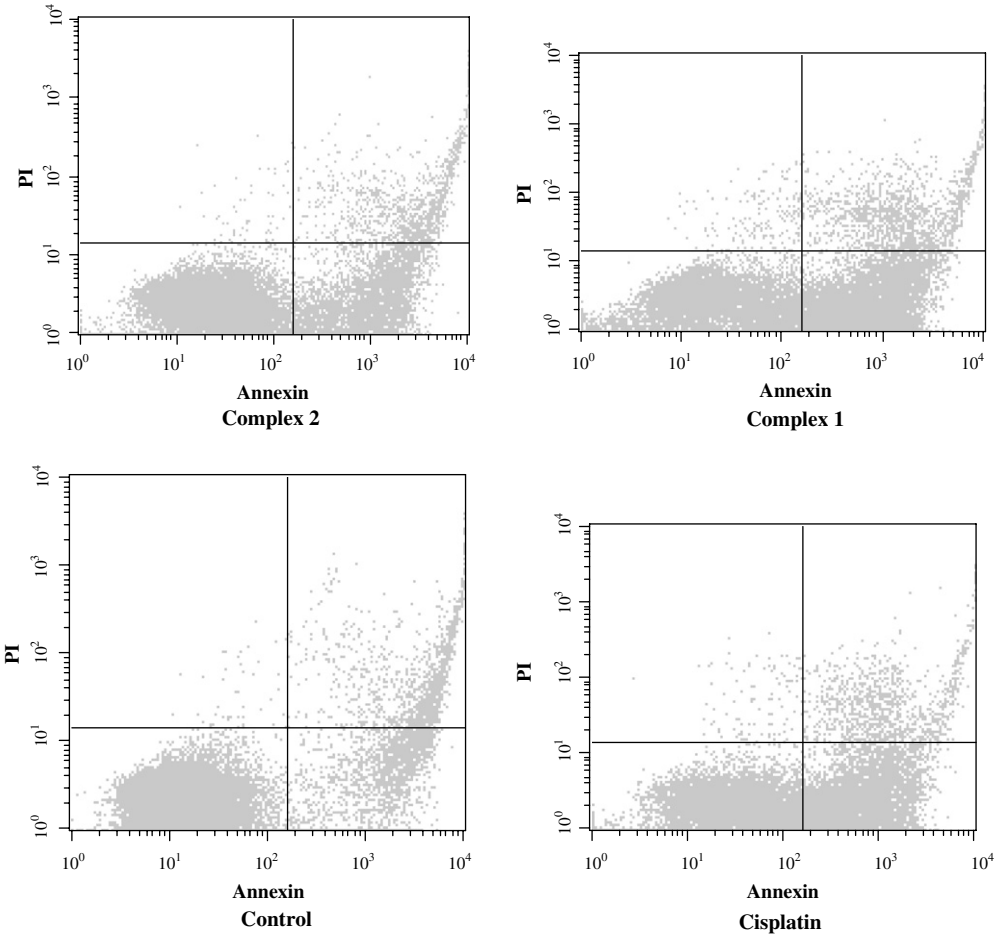


Figure 8. Images of flow cytometry diagrams for compounds **1**, **2**, cisplatin and control blank.

In vitro studies of antiproliferative activity were performed against HL-60 cancer cell line (leukaemia). Moreover, flow cytometry studies allowed us to evaluate the death mechanism induced by the platinum complexes. In all biochemical assays we have observed changes in the structure of DNA similar to that induced by cisplatin, drug used as a reference or standard in our studies. This behaviour was confirmed by the in vitro cytotoxicity tests. It is worth noting that the IC_{50} value of compound **1** is half of the IC_{50} value for **2** at 24 h of incubation. In other words, **1** is twice as active as **2** for such incubation period. Finally, by flow cytometry it was observed that **1** induced apoptosis at a level considerably higher than **2** and even than cisplatin and a low level of necrosis, which is desirable in an antiproliferative drug. Summarizing, there is a clear difference of behaviour between enantiomers **1** and **2** observed by all techniques and in all assays carried out. The reasons, at the molecular level, for this phenomenon are under study in our laboratory.

4. Experimental

4.1. Materials and methods

Reactions that required an inert atmosphere were conducted under dry nitrogen or argon and the glassware was oven-dried (120 °C). THF, Et_2O and benzene were distilled from sodium/benzophenone prior to use. CH_2Cl_2 was dried by refluxing it over CaH_2 under nitrogen. *Elemental analyses* (C, H, N, S) were carried out on a Carlo Erba EA1108 apparatus. *Infrared spectra* were recorded on a FT-IR NICOLET 510 spectrophotometer in a 4000–400 cm^{-1} range. The oily samples were recorded as films on NaCl plates and in the case of solids as KBr plates. *NMR spectra* were obtained on a Varian Gemini-200, a Varian Unity-300 plus or a Varian VXR-500, using $CDCl_3$, [D_7]DMF or [D_6]DMSO as solvents. ^1H NMR spectra were obtained at 200 MHz, 300 MHz or 400 MHz frequencies and chemical shifts are given in ppm, relative to tetramethylsilane (TMS). ^{13}C NMR and DEPT experiments were recorded at 50 MHz, 75 MHz or 100 MHz and were referenced to the 77.0 ppm resonance of $CDCl_3$. *Mass spectra* were obtained by a Fisons VG Quattro triple quadrupole analyzer in the 1800–200 *m*/*z* range, fitted with a canon working at 10 kV. The mass spectra were carried out using MeCN–H_2O as solvent under electrospray (ESP-MS) conditions or by the FAB(+) technique using NBA as a matrix. In this last technique, the samples were dissolved in DMSO and bombarded with accelerated caesium atoms. In the case of working with a chemical ionization technique, a Hewlett–Packard 5890 mass spectrometer was used (the operating conditions are specified for each case). *Melting points* were measured on a Galenkamp and on a Stuart Scientific SMP3 apparatus. *Circular dichroism* (CD) spec-

tra were obtained at room temperature on a JASCO J720 spectropolarimeter with a 450 W xenon lamp using a computer for spectral subtraction and noise reduction. Each sample was scanned three times in a range of wavelength between 220 and 360 nm at a 50 nm min^{-1} rate. The CD spectra obtained are the mean of the three independent scans. The data are expressed as mean residue molar ellipticity Θ in deg cm^2 mol^{-1}. *Atomic Force Microscopy* (AFM). The samples were imaged in a Nanoscope III Multimode atomic force microscope (Digital Instrumentals Inc., Santa Barbara, CA) operating in tapping mode in air, at a scan rate of 1–3 Hz. The microscope probes were 125-μm long monocrystalline silicon cantilevers with integrated conical-shaped Si tips (Nanosensors GmbH, Germany) with an average resonance frequency $f_0 \approx 330$ kHz and spring constant $K \approx 50$ N m^{-1}. The cantilever is rectangular and the tip radius given by supplier is ≈5 nm, with a 35° cone angle and a high aspect ratio. In general the images were obtained at room temperature (23 ± 2 °C) and the relative humidity was typically 55%.

4.2. Synthesis of *cis*-[(1*S*,2*R*,3*S*)-1,7,7-trimethylbicyclo-[2.2.1]heptane-2,3-diamine]dichloroplatinum(II) (1)

In a 10 mL cone-shaped flask, diamine **3** hydrochloride (0.066 g, 0.27 mmol) was dissolved in H_2O (2.5 mL) under nitrogen atmosphere. In other flask, a solution of K_2PtCl_4 (0.113 g, 0.27 mmol) in H_2O (1 mL) was prepared under nitrogen. The aqueous diamine solution was transferred via cannula to the second solution. The mixture was stirred for 1 h at room temperature and it was kept to stand in the dark at room temperature for 24 h. The formed brown solid was filtered out by using a sintered-glass plate (10–16 μm of pore size) and it was dried in vacuo and afterwards into a desiccator in the presence of P_2O_5. Yield: 0.061 g, 51%.

IR (KBr): ν_{max} 3506, 3215 (νNH_2), 2958, 2879 ($\nu C^3_{sp} - H$), 1593 (δNH_2), 1460 ($\delta C^3_{sp} - H$) cm^{-1}. ^1H NMR (400 MHz, [D_6]DMSO, 25 °C, TMS): δ 0.67 (s, 3 H; H-1^{IV} or H-1^{V}), 0.85–0.89 (m, 1H; H-6 b), 0.89 (s, 3 H; H-1^{IV} or H-1^{V}), 0.92–0.97 (m, 1H; H-5 b), 1.18 (s, 3 H; H-1′), 1.25–1.33 (m, 1H; H-6a), 1.40–1.51 (m, 1H; H-5a), 1.73–1.74 (d, J_{4-5a} = 4.4 Hz, 1H; H-4), 2.81–2.87 (m, 1H; H-2), 2.93–2.99 (m, 1H; H-3), 4.22–4.29 (m, 1H; H1″ or H-2″), 4.66–4.73 (m, 1H; H-1‴ or H-2‴), 5.75–5.80 (m, 1H; H-1″ or H-2″), 5.83–5.89 (m, 1H; H-1‴ or H-2‴) ppm. ^{13}C NMR (100 MHz, [D_7]DMF, 25 °C): δ 10.8 (C-1^{IV} or C-1^{V}), 20.8 (C-1^{IV} or C-1^{V}), 21.3 (C-1′), 26.3 (C-5), 35.2 (C-6), 47.5 (C-7), 50.2 (C-4), 51.3 (C-1), 70.8 (C-3), 75.1 (C-2) ppm. MS [FAB(+), NBA]: *m/z* (%) 476.8 (83) [M–Cl^-+DMSO], 397.8 (100) [M–Cl^-–H^+], 362.9 (56) [M–2Cl^-–H^+]. Anal. Calcd for $C_{10}H_{20}Cl_2N_2Pt$: C, 27.7; H, 4.6; N, 6.4. Found: C, 27.9; H, 4.5; N, 6.0.

4.3. Synthesis of *cis*-[(1*R*,2*S*,3*R*)-1,7,7-trimethylbicyclo[2.2.1]heptane-2,3-diamine]dichloroplatinum(II) (2)

Substrate **4** (0.041 g, 0.17 mmol) and K_2PtCl_4 (0.079 g, 0.19 mmol) were submitted to the same experimental conditions as that in the preceding procedure, obtaining a light brown solid. Yield: 0.042 g, 57%. The ^1H NMR, ^{13}C NMR and IR spectra were, as expected, identical to those of enantiomer **1**. MS [FAB(+), NBA,]: *m/z* (%) 397.8 (100) [M–Cl–H^+], 434.3 (43) [M], 513.4 (64) [M+DMSO+H^+]. Anal. Calcd for $C_{10}H_{20}Cl_2N_2Pt$: C, 27.7; H, 4.6; N, 6.4. Found: C, 28.0; H, 4.7; N, 6.3.

4.4. Synthesis of (1*S*,2*R*,3*S*)-1,7,7-trimethylbicyclo[2.2.1]-heptane-2,3-diammonium dichloride (3)

In a 25-mL round-bottomed flask, hydrochlorides **11** and/ or **12** (0.279 g, 0.66 mmol) and ammonium formate (0.601 g, 9.53 mmol) were dissolved in anhydrous MeOH (14 mL) under nitrogen atmosphere and the resulting solution was purged five times by N_2/vacuum cycles to eliminate the traces of oxygen dissolved in methanol. On the other hand, in a three-necked 50-mL flask, fitted with septa, 10% Pd/C (0.126 g, 45% w/w) was weighed under argon. Next a Dimroth condenser was adapted and the system pumped out and back filled with nitrogen five times. The solution of **11** (or **12**) and ammonium formate in methanol was transferred via cannula to the second flask and the mixture was purged, as mentioned before, 10 times. The reaction mixture was heated to reflux of solvent for 1.25 h (control by TLC). The crude mixture was let to cool down to room temperature and it was filtered through a two-layers short pad of Celite® and activated neutral alumina. The resulting clear solution was concentrated to dryness in vacuo, obtaining a white solid. The resulting solid was dissolved in 2 M HCl (25 mL) to generate the corresponding hydrochloride. The aqueous solution was extracted with ethyl acetate (2 × 15 mL) to remove dibenzoyl. The resulting aqueous solution was concentrated to dryness in vacuo obtaining **3** as a pure white solid. Yield: 0.106 g, 85%. $[\alpha]_D^{23}$ + 27.1 (*c* = 0.98, MeOH). IR (KBr): ν_{max} 3434 (νNH_2), 2957 ($\nu C^3_{sp} - H$), 3000–2000 (νNH_3^+), 1514 (δNH_3^+), 1461 ($\delta C^3_{sp} - H$) cm^{-1}. ^1H NMR (400 MHz, D_2O, 25 °C): δ 0.80 (s, 3 H; H-1″ or H-1‴), 0.93 (s, 3H; H-1″ or H-1‴), 0.95 (s, 3H; H-1′), 0.99–1.24 (m, 2H; H-5b, H-6b), 1.56–1.63 (m,1H; H-6a), 1.75–1.90 (m, 1H; H-5a), 2.07–2.08 (d, J_{4-5a} = 4.8 Hz, 1H; H-4), 3.54–3.56 (d, J_{2-3} = 9.2 Hz, 1H; H-2), 3.62–3.65 (d, J_{3-2} = 9.2 Hz, 1H; H-3) ppm. ^{13}C NMR (100 MHz, D_2O, 25 °C): δ 10.6 (C-1″ or C-1‴), 20.1 (C-1″ or C-1‴), 20.5 (C-1′), 25.7 (C-5), 34.6 (C-6), 47.6 (C-7), 49.0 (C-4), 49.2 (C-1), 56.6 (C-3), 60.4 (C-2) ppm. MS (EI, 70 eV): *m/z* (%) 168.9 (100) [M–2HCl]. Anal. Calcd for $C_{10}H_{22}Cl_2N_2$: C, 49.80; H, 9.19; N, 11.61. Found: C, 49.93; H, 9.21; N, 11.58.

4.5. Synthesis of (1*R*,2*S*,3*R*)-1,7,7-trimethylbicyclo[2.2.1]-heptane-2,3-diammonium dichloride (4)

Following an experimental procedure similar to the previously described, substrates **17** and/ or **18** (0.210 g, 0.50 mmol) were reacted with ammonium formate (0.451 g, 7.22 mmol) and 10% Pd/C (0.095 g, 45% w/w) in MeOH (12 mL), obtaining product **4** as a pure white solid. Yield: 0.109 g, 90%. $[\alpha]_D^{23}$ − 26.8 (*c* = 0.21, MeOH). Anal. Calcd for $C_{10}H_{22}Cl_2N_2$: C, 49.80; H, 9,19; N, 11.61. Found: C, 49.73; H, 9.25; N, 11.68. The ^1H NMR, ^{13}C NMR, IR and MS spectra of **4** were identical to those of enantiomer **3**.

4.6. Preparation of (±)-1,2-diphenylethane-1,2-diamine, (±)-DPEDA (5)

This compound was prepared according to the synthetic methodology described in Ref. 27.

4.7. Synthesis of 6, 7 and of intermediate 8

In a 250-mL round-bottomed flask, fitted with a Dean–Stark apparatus, (1*S*)-camphorquinone (1 g, 6.02 mmol) and (±)-DPEDA (**5**) (1.277 g, 6.02 mmol) dissolved in anhydrous benzene (140 mL) were placed, under nitrogen atmosphere. The solution was heated to reflux of solvent, under inert atmosphere for 17 h (control by TLC). The reaction mixture was let to reach room temperature and it was concentrated to dryness. The resulting crude product was submitted to flash column chromatography on silica gel, eluting with mixtures of hexane/EtOAc/MeOH of increasing polarity, isolating the following fractions: 0.497 g of **8** (hexane/AcOEt 85:15), 1.098 g of **6** (hexane/AcOEt 85:15 y 8:2) and 0.096 g of **7** (hexane/AcOEt 75:25 and 7:3) as yellowish oils.

4.7.1. (1*S*,4*R*,5*R*)-4,5-diphenyl-1,11,11-trimethyl-3,6-diazatricyclo[6.2.1.0^{2,7}]undeca-2,6-diene (6). IR (film): ν_{max} 3062, 3033 ($\nu C^2_{sp}-H$), 2961, 2879 ($\nu C^3_{sp}-H$), 1654 ($\nu C^2_{sp}-N$), 1452 ($\delta C^3_{sp}-H$) cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C): δ 0.92 (s, 3 H; H-12), 1.05 (s, 3H; H-13 or H-14), 1.14 (s, 3H; H-13 or H-14), 1.62–1.65 (m, 2H; H-10), 1.86–1.92 (m, 1H; H-9b), 2.17–2.24 (m, 1H; H-9a), 2.59 (d, J_{8-9a} = 4.5 Hz, 1H; H-8), 4.38 (d, J_{5-4} = 11 Hz, 1H; H-4 or H-5), 4.41 (d, J_{5-4} = 11 Hz, 1H; H-4 or H-5), 6.92–6.96 (m, 4H; H-3′, H-5′, H-3″, H-5″), 7.18–7.22 (m, 6H; H-2′, H-4′, H-6′, H-2″, H-4″, H-6″) ppm. ^{13}C NMR (50 MHz, $CDCl_3$, 25 °C): δ9.9 (C-12), 17.7 (C-13 or C-14), 20.6 (C-13 or C-14), 25.4 (C-9), 31.6 (C-10), 46.4 (C-11), 52.7 (C-8), 53.0 (C-1), 65.0 (C-5), 67.2 (C-4), 126.9 (C-4′ or C-4″), 127.0 (C-4′ or C-4″), 127.6 (C-2′, C-6′ or C-2″, C-6″), 127.7 (C-2′, C-6′ or C-2″, C-6″), 128.1 (C-3′, C-5′ or C-3″, C-5″), 142.3 (C-1′ or C-1″), 142.4 (C-1′ or C-1″), 165.8 (C-2 or C-7), 167.8 (C-2 or C-7) ppm. MS (EI, 70 eV): *m*/*z* (%) 343 (27) [M+H$^+$], 342 (100) [M], 341 (24) [M–H$^+$], 340 (59) [M–2H$^+$]. Anal. Calcd for C24H$_{26}$N$_2$: C, 84.17; H, 7.65; N, 8.18. Found: C, 84.15; H, 7.67; N, 8.20.

4.7.2. (1*S*,4*S*,5*S*)-4,5-diphenyl-1,11,11-trimethyl-3,6-diazatricyclo[6.2.1.0^{2,7}]undeca-2,6-diene (7). IR (film): ν_{max} 3062, 3033 ($\nu C^2_{sp}-H$), 2961, 2879 cm^{-1} ($\nu C^3_{sp}-H$), 1654 cm^{-1} ($\nu C^2_{sp}-N$), 1452 cm^{-1} ($\delta C^3_{sp}-H$) cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C): δ 0.91 (s, 3 H; H-12), 1.08 (s, 3H; H-13 or H-14), 1.17 (s, 3H; H-13 or H-14), 1.81 (qd, J_{10-9a} = 10 Hz, J_{10-12} = 2.5 Hz, 2H; H-10), 1.97–2.03 (m, 1H; H-9b), 2.19–2.24 (m, 1H; H-9a), 2.62 (d, J_{8-9a} = 4.5 Hz, 1H; H-8), 4.94 (d, J_{4-5} = 8 Hz, 1H; H-4 or H-5), 4.98 (d, J_{4-5} = 8.5 Hz, 1H; H-4 or H-5), 6.74–6.76 (m, 2H; H-3′, H-5′ or H-3″, H-5″), 6.82–6.84 (m, 2H; H-3′, H-5′ or H-3″, H-5″), 7.05–7.07 (m, 6H; H-2′, H-4′, H-6′, H-2″, H-4″, H-6″) ppm. ^{13}C NMR (50 MHz, $CDCl_3$, 25 °C): δ 9.9 (C-12), 17.5 (C-13 or C-14), 20.7 (C-13 or C-14), 24.5 (C-9), 32.5 (C-10), 47.0 (C-11), 53.0 (C-8), 53.2 (C-1), 63.4 (C-5), 63.7 (C-4), 126.7 (C-4′ or C-4″), 126.9 (C-4′ or C-4″), 127.6 (C-2′, C-6′ or C-2″, C-6″), 127.8 (C-2′, C-6′ or C-2″, C-6″), 127.9 (C-3′, C-5′ or C-3″, C-5″), 128.1 (C-3′, C-5′ or C-3″, C-5″), 137.2 (C-1′ or C-1″), 138.1 (C-1′ or C-1″), 168.3 (C-2 or C-7), 169.5 (C-2 or C-7) ppm. MS (EI, 70 eV): *m*/*z* (%) = 343 (27) [M+H$^+$], 342 (100) [M], 341 (24) [M–H$^+$], 340 (59) [M–2H$^+$]. Anal. Calcd for C$_{24}$H$_{26}$N$_2$: C, 84.17; H, 7.65; N, 8.18. Found: C, 84.21; H, 7.62; N, 8.13.

4.7.3. (2*S*,8*S*)-4,5-diphenyl-8,11,11-trimethyl-3,6-diazatricyclo[6.2.1.0^{2,7}]undec-6-en-2-ol (8). IR (film): ν_{max} 3344 (νO-H), 3030 ($\nu C^2_{sp}-H$), 2960 ($\nu C^3_{sp}-H$), 1746 ($\nu C^2_{sp}-N$), 1453 ($\delta C^3_{sp}-H$) cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 0.99 (s, 3H; H-12), 1.05 (s, 3H; H-13 or H-14), 1.17 (s, 3H; H-13 or H-14), 1.41–1.48 (m, 1H; H-9), 1.65–1.72 (m, 1H; H-9), 1.78–1.87 (m, 1H; H-10), 2.06–2.07 (d, J_{1-10a} = 4 Hz, 1H; H-1), 2.17–2.24 (m, 1H; H-10), 2.47 (s, 1H; H-1‴), 3.94–3.96 (d, J_{5-4} = 9.2 Hz, 1H; H-5), 4.48–4.50 (d, J_{4-5} = 9.2 Hz, 1H; H-4), 7.21–7.26 (m, 4H; H-3′, H-5′, H-3″, H-5″), 7.28–7.32 (m, 2H; H-4′, H-4″), 7.36–7.38 (m, 4H; H-2′, H-6′, H-2″, H-6″) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ 9.7 (C-12), 20.4 (C-13 or C-14), 22.9 (C-13 or C-14), 23.0 (C-10), 31.3 (C-9), 45.1 (C-11), 55.7 (C-1), 59.0 (C-8), 70.1 (C-5), 71.2 (C-4), 84.6 (C-2), 127.1 (C-4′ or C-4″), 127.2 (C-4′ or C-4″), 127.7 (C-2′, C-6′ or C-2″, C-6″), 127.8 (C-2′, C-6′ or C-2″, C-6″), 128.7 (C-3′, C-5′ or C-3″, C-5″), 128.9 (C-3′, C-5′ or C-3″, C-5″), 139.7 (C-1′ or C-1″), 140.5 (C-1′ or C-1″), 166.3 (C-7) ppm. MS (EI, 70 eV): *m*/*z* (%) = 361.1 (100) [M+H$^+$], 343.1 (28) [M–OH]$^+$]. Anal. Calcd for C$_{25}$H$_{32}$N$_2$O: C, 79.75; H, 8.57; N, 7.44. Found: C,79.80; H, 8.55; N, 7.39.

4.8. Synthesis of 9 and 10

In a 25-mL flask, fitted with septa and a magnetic stirring bar, substrate **6** (or **7**) (0.459 g, 1.34 mmol) was dissolved in anhydrous methanol (16 mL) under argon atmosphere. The solution was cooled down to 0 °C in an ice bath and sodium borohydride (0.259 g, 6.83 mmol) was added at once. After addition the system was purged with argon, the cooling bath was removed and the reaction mixture was stirred for 1 h at room temperature (controlled by TLC). To generate the hydrochloride, 2 M HCl was added (15 mL), and methanol was removed by the rotary evaporator. The resulting aqueous solution was extracted with EtOAc (4 × 10 mL). The organic phases were combined together, dried on anhydrous magnesium sulfate, filtered and concentrated to dryness, resulting in 0.276 g of dark oil mainly formed by non-reacted substrate **6** (or **7**). The aqueous phase was treated with 2.5 M NaOH (20 mL) to regenerate the free amines which formed a white precipitate. EtOAc (10 mL) was added to dissolve the solid and the aqueous phase was extracted with more EtOAc (4 × 10 mL). All organic phases were combined together, dried on anhydrous magnesium sulfate, filtered and concentrated to dryness, resulting in 0.207 g of colourless oil corresponding to product **9** (or **10**). The fraction of dark oil corresponding to non-converted substrate **6** (or **7**) was reacted a second time with $NaBH_4$ (0.155 g, 4.10 mmol), under the same previous reaction conditions, affording additional 0.072 g of **9** (or **10**). Overall yield: 0.279 g, 99.9%.

4.8.1. (1*S*,2*R*,4*R*,5*R*,7*S*)-4,5-diphenyl-1,11,11-trimethyl-3,6-diazatricyclo[6.2.1.0^{2,7}]undecane (9). IR (film): ν_{max} 3062, 3031 (νC^2_{sp} – H, 2927 (νC^3_{sp} – H), 1491 (νC^3_{sp} – N), 1452 (δC^3_{sp} – H) cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 0.88 (s, 3 H; H-13 or H-14), 0.90 (s, 3H; H-13 or H-14), 1.03–1.10 (m, 2H; H-10), 1.48–1.59 (m, 2H; H-9), 1.69 (s, 3H; H-12), 1.78–1.90 (m, 3H; H-3, H-6, H-8), 2.88 (d, J_{7-2} = 7.3 Hz, 1H; H-2 or H-7), 3.16 (d, J_{2-7} = 7.3 Hz, 1H; H-2 or H-7), 3.60 (d, J_{4-5} = 8.4 Hz, 1H; H-4 or H-5), 4.06 (d, J_{4-5} = 8.4 Hz, 1H; H-4 or H-5), 7.21–7.23 (m, 2H; H-4′, H-4″), 7.26 (m, 4H; H-2′, H-6′, H-2″, H-6″), 7.25–7.27 (m, 4H; H-3′, H-5′, H-3″, H-5″) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ 11.4 (C-13 or C-14), 21.8 (C-12), 22.8 (C-13 or C-14), 27.5 (C-10), 35.7 (C-9), 47.4 (C-1 or C-11), 48.5 (C-1 or C-11), 50.8 (C-8), 58.3 (C-2 or C-7), 63.9 (C-4 or C-5), 65.9 (C-2 or C-7), 70.7 (C-4 or C-5), 126.8 (C-4′ or C-4″), 127.5 (C-4′ or C-4″), 127.6 (C-2′, C-2″ or C-6′, C-6″), 127.8 (C-2′, C-2″ or C-6′, C-6″), 128.3 (C-3′, C-3″ or C-5′, C-5″), 128.6 (C-3′, C-3″ or C-5′, C-5″), 144.2 (C-1′ or C-1″), 144.9 (C-1′ or C-1″) ppm. MS (EI, 70 eV): *m*/*z* (%) = 347 (27) [M+H$^+$], 346 (100) [M], 342 (32) [M–4H$^+$]. Anal. Calcd for $C_{24}H_{30}N_2$: C, 83.19; H, 8.73; N, 8.08. Found: C, 83.23; H, 8.77; N, 8.12.

4.8.2. (1*S*,2*R*,4*S*,5*S*,7*S*)-4,5-diphenyl-1,11,11-trimethyl-3,6-diazatricyclo[6.2.1.0^{2,7}]undecane (10). IR (film): ν_{max} 3062, 3031 (νC^2_{sp} – H), 2927 (νC^3_{sp} – H), 1491 (νC^3_{sp} – N), 1452 (δC^3_{sp} – H) cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 0.86 (s, 3H; H-13 or H-14), 0.96 (s, 3H; H-13 or H-14), 1.05–1.21 (m, 2H; H-10), 1.52–1.74 (m, 2H; H-9), 1.65 (s, 3H; H-12), 1.75–1.80 (m, 1H; H-8), 1.95–2.10 (m, 1H; H-3 or H-6), 2.26–2.39 (m, 1H; H-3 or H-6), 2.97 (d, J_{7-2} = 7.2 Hz, 1H; H-2 or H-7), 3.02 (d, J_{2-7} = 7.3 Hz, 1H; H-2 or H-7), 3.65 (d, J_{4-5} = 7.9 Hz, 1H; H-4 or H-5), 4.18 (d, J_{5-4} = 7.9 Hz, 1H; H-4 or H-5), 7.16–7.24 (m, 2H; H-4′, H-4″), 7.25 (m, 4H; H-2′, H-6′, H-2″, H-6″), 7.26–7.27 (m, 4H; H-3′, H-5′, H-3″, H-5″) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ 12.1 (C-13 or C-14), 21.6 (C-12), 23.1 (C-13 or C-14), 27.0 (C-10), 36.4 (C-9), 47.5 (C-1 or C-11), 48.4 (C-1 or C-11), 50.6 (C-8), 61.5 (C-2 or C-7), 63.0 (C-4 or C-5), 65.9 (C-2 or C-7), 66.3 (C-4 or C-5), 126.8 (C-4′ or C-4″), 127.4 (C-4′ or C-4″), 127.5 (C-2′, C-2″ or C-6′, C-6″), 127.7 (C-2′, C-2″ or C-6′, C-6″), 128.4 (C-3′, C-3″ or C-5′, C-5″), 128.7 (C-3′, C-3″ or C-5′, C-5″), 144.5 (C-1′ or C-1″), 145.0 (C-1′ or C-1″) ppm. MS (EI, 70 eV): *m*/*z* (%) = 347 (33) [M+H$^+$], 346 (100) [M], 342 (32) [M–4H$^+$]. Anal. Calcd for $C_{24}H_{30}N_2$: C, 83.19; H, 8.73; N, 8.08. Found: C, 83.17; H, 8.79; N, 8.03.

4.9. Synthesis of 11 and 12

Compounds **9** and/or **10** were treated with 2 M HCl and the resulting hydrochlorides **11** and/or **12** were formed. The aqueous solution was concentrated to dryness in a dry ice rotary evaporator, affording products **11** and/or **12** as yellowish solids in quantitative yield.

4.9.1. (1*S*,2*R*,4*R*,5*R*,7*S*)-4,5-diphenyl-1,11,11-trimethyl-3,6-diazatricyclo[6.2.1.0^{2,7}]undecane dihydrochloride (11). IR (KBr): ν_{max}3348 (νN–H), 3041 (νC^2_{sp} – H), 2968 (νC^3_{sp} – H), 2958–2387 (νNH^+_2), 1570 (δN–H), 1451 (δC^3_{sp} – H) cm^{-1}. ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 0.84 (s, 3 H; H-13 or H-14), 0.95 (s, 3H; H-13 or H-14), 1.15–1.30 (m, 2H; H-10), 1.23 (s, 3H; H-12), 1.58–1.72 (m, 1H; H-9a or H-9b), 1.78–1.90 (m, 1H; H-9a or H-9b), 2.02 (d, J_{8-9a} = 4.0 Hz, 1H; H-8), 3.82 (d, J_{2-7} = 8.4 Hz, 1H; H-2 or H-7), 3.92 (d, J_{2-7} = 8.4 Hz, 1H; H-2 or H-7), 5.00 (d, J_{4-5} = 11.6 Hz, 1H; H-4 or H-5), 5.04 (d, J_{4-5} = 11.2 Hz, 1H; H-4 or H-5), 7.20–7.22 (m, 2H; H-4′, H-4″), 7.24–7.26 (m, 4H; H-2′, H-6′, H-2″, H-6″), 7.29–7.34 (m, 4H; H-3′, H-5′, H-3″, H-5″) ppm. ^{13}C NMR (100 MHz, D_2O, 25 °C): δ 10.6 (C-12), 20.5 (C-13 or C-14), 21.2 (C-13 or C-14), 26.3 (C-9), 34.5 (C-10), 48.1 (C-11), 48.8 (C-1), 49.0 (C-8), 56.0 (C-7), 59.5 (C-4 or C-5), 60.2 (C-4 or C-5), 66.7 (C-2), 128.5 (C-4′, C-4″), 128.8 (C-2′, C-2″ or C-6′, C-6″), 128.9 (C-2′, C-2″ or C-6′, C-6″), 129.6 (C-3′, C-3″ or C-5′, C-5″), 129.7 (C-3′, C-3″ or C-5′, C-5″), 142.3 (C-1′ or C-1″), 142.5 (C-1′ or C-1″) ppm. MS-FAB [FAB(+), NBA,]: *m*/*z* (%) 349 (47) [(M–2HCl)+2H$^+$], 348 (71) [(M–2HCl)+H$^+$], 347 (100) [M–2HCl]. Anal. Calcd for $C_{24}H_{32}Cl_2N_2$: C, 68.73; H, 7.69; N, 6.68. Found: C, 68.80; H, 7.65; N, 6.71.

4.9.2. (1*S*,2*R*,4*S*,5*S*,7*S*)-4,5-diphenyl-1,11,11-trimethyl-3,6-diazatricyclo[6.2.1.0^{2,7}]undecane dihydrochloride (12). IR (KBr): ν_{max} 3348 (νN–H), 3041 (νC^2_{sp} – H), 2968 (νC^3_{sp} – H), 2958–2387 (νNH^+_2), 1570 (δN–H), 1451 (δC^3_{sp} – H) cm^{-1}. ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 0.86 (s, 3H; H-13 or H-14), 0.95 (s, 3H; H-13 or H-14), 1.15–1.30 (m, 2H; H-10), 1.26 (s, 3H; H-12), 1.58–1.72 (m, 1H; H-9a or H-9b), 1.78–1.90 (m, 1H; H-9a or H-9b), 1.98 (d, J_{8-9a} = 4.0 Hz, 1H; H-8), 3.82 (d, J_{2-7} = 8.4 Hz, 1H; H-2 or H-7), 3.92 (d, J_{2-7} = 8.4 Hz, 1H; H-2 or H-7), 4.83 (d, J_{4-5} = 6.4 Hz, 1H; H-4 or H-5), 4.87 (d, J_{4-5} = 6.0 Hz, 1H; H-4 or H-5), 7.20–7.22 (m, 2H; H-4′, H-4″), 7.24–7.26 (m, 4H; H-2′, H-6′, H-2″, H-6″), 7.29–7.34 (m, 4H; H-3′, H-5′, H-3″, H-5″) ppm. ^{13}C NMR (100 MHz, D_2O, 25 °C): δ 10.1 (C-12), 20.5 (C-13 or C-14), 21.3 (C-13 or C-14), 25.4 (C-9), 35.0 (C-10), 47.8 (C-11), 47.9 (C-1), 49.7 (C-8), 56.9 (C-7), 59.2 (C-4 or C-5), 59.8 (C-4 or C-5), 66.7 (C-2), 128.3 (C-4′, C-4″), 128.9 (C-2′, C-2″ or C-6′, C-6″), 129.4 (C-2′, C-2″ or C-6′, C-6″), 129.5 (C-3′, C-3″ or C-5′, C-5″), 129.8 (C-3′, C-3″ or C-5′, C-5″), 140.1 (C-1′ or C-1″), 140.8 (C-1′ or C-1″) ppm. MS-FAB [FAB(+), NBA,]: *m*/*z* (%) 349 (47) [(M–2HCl)+2H$^+$], 348 (71) [(M–2HCl)+H$^+$], 347 (100) [M–2HCl]. Anal. Calcd for $C_{24}H_{32}Cl_2N_2$: C, 68.73; H, 7.69; N, 6.68. Found: C, 68.70; H, 7.71; N, 6.66.

4.10. Synthesis of 13 and 14

Following the same experimental procedure previously described for the synthesis of **6** and **7**, (1*R*)-camphorquinone (0.501 g, 2.98 mmol) was reacted with (±)-DPEDA (0.633 g, 2.98 mmol) in anhydrous benzene (70 mL). The crude product was submitted to flash column chromatography, isolating 0.764 of **13** (with hexane/AcOEt 85:15) and 0.110 g of diastereoisomer **14** (with hexane/AcOEt 85:15 and 8:2). Yield: 85%.

4.10.1. (1*R*,4*S*,5*S*)-4,5-diphenyl-1,11,11-trimethyl-3,6-diazatricyclo[6.2.1.0^{2,7}]undeca-2,6-diene (13). IR (film): ν_{max} 3062, 3033 (νC^2_{sp} – H), 2961, 2879 (νC^3_{sp} – H), 1654 (νC^2_{sp} – N), 1452 (δC^3_{sp} – H) cm^{-1}. ^{1}H NMR (500 MHz,

$CDCl_3$, 25 °C): δ 0.92 (s, 3H; H12), 1.05 (s, 3H; H-13 or H-14), 1.14 (s, 3H; H-13 or H-14), 1.62–1.65 (m, 2H; H-10), 1.86–1.92 (m, 1H; H-9b), 2.17–2.24 (m, 1H; H-9a), 2.59 (d, J_{8-9a} = 4.5 Hz, 1H; H-8), 4.38 (d, J_{5-4} = 11 Hz, 1H; H-4 or H-5), 4.41 (d, J_{5-4} = 11 Hz, 1H; H-4 or H-5), 6.92–6.96 (m, 4H; H-3′, H-5′, H-3″, H-5″), 7.18–7.22 (m, 6H; H-2′, H-4′, H-6′, H-2″, H-4″, H-6″) ppm. ^{13}C NMR (50 MHz, $CDCl_3$, 25 °C): δ 9.9 (C-12), 17.7 (C-13 or C-14), 20.6 (C-13 o C-14), 25.4 (C-9), 31.6 (C-10), 46.4 (C-11), 52.7 (C-8), 53.0 (C-1), 65.0 (C-5), 67.2 (C-4), 126.9 (C-4′ or C-4″), 127.0 (C-4′ or C-4″), 127.6 (C-2′, C-6′ or C-2″, C-6″), 127.7 (C-2′, C-6′ or C-2″, C-6″), 128.1 (C-3′, C-5′ or C-3″, C-5″), 142.3 (C-1′ or C-1″), 142.4 (C-1′ or C-1″), 165.8 (C-2 or C-7), 167.8 (C-2 or C-7) ppm. MS (EI, 70 eV): *m*/*z* (%) = 343 (27) [M+H$^+$], 342 (100) [M], 341 (24) [M–H$^+$], 340 (59) [M–2H$^+$]. Anal. Calcd for $C_{24}H_{26}N_2$: C, 84.17; H, 7.65; N, 8.18. Found: C, 84.23; H, 7.61; N, 8.15.

4.10.2. (1*R*,4*R*,5*R*)-4,5-diphenyl-1,11,11-trimethyl-3,6-diazatricyclo[6.2.1.0^{2,7}]undeca-2,6-diene (14). IR (film): ν_{max} 3062, 3033 (νC^2_{sp} – H), 2961, 2879 (νC^3_{sp} – H), 1654 (νC^2_{sp} – N), 1452 (δC^3_{sp} – H) cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C): δ 0.91 (s, 3H; H-12), 1.08 (s, 3H; H-13 or H-14), 1.17 (s, 3H; H-13 or H-14), 1.81 (qd, J_{10-9a} = 10 Hz, J_{10-12} = 2.5 Hz, 2H; H-10), 1.97–2.03 (m, 1H; H-9b), 2.19–2.24 (m, 1H; H-9a), 2.62 (d, J_{8-9a} = 4.5 Hz, 1H; H-8), 4.94 (d, J_{4-5} = 8 Hz, 1H; H-4 or H-5), 4.98 (d, J_{4-5} = 8.5 Hz, 1H; H-4 or H-5), 6.74–6.76 (m, 2H; H-3′, H-5′ or H-3″, H-5″), 6.82–6.84 (m, 2H; H-3′, H-5′ or H-3″, H-5″), 7.05–7.07 (m, 6H; H-2′, H-4′, H-6′, H-2″, H-4″, H-6″) ppm. ^{13}C NMR (50 MHz, $CDCl_3$, 25 °C): δ 9.9 (C-12), 17.5 (C-13 or C-14), 20.7 (C-13 or C-14), 24.5 (C-9), 32.5 (C-10), 47.0 (C-11), 53.0 (C-8), 53.2 (C-1), 63.4 (C-5), 63.7 (C-4), 126.7 (C-4′ or C-4″), 126.9 (C-4′ or C-4″), 127.6 (C-2′, C-6′ or C-2″, C-6″), 127.8 (C-2′, C-6′ or C-2″, C-6″), 127.9 (C-3′, C-5′ or C-3″, C-5″), 128.1 (C-3′, C-5′ or C-3″, C-5″), 137.2 (C-1′ or C-1″), 138.1 (C-1′ or C-1″), 168.3 (C-2 or C-7), 169.5 (C-2 or C-7) ppm. MS (EI, 70 eV): *m*/*z* (%) = 343 (27) [M+H$^+$], 342 (100) [M], 341 (24) [M–H$^+$], 340 (59) [M–2H$^+$]. Anal. Calcd for $C_{24}H_{26}N_2$: C, 84.17; H, 7.65; N, 8.18. Found: C, 84.13; H, 7.60; N, 8.19.

4.11. Synthesis of 15 and 16

The preparation of compounds **15** and **16** was carried out by the same procedure previously described for products **9** and **10**. Thus, substrates **13** and/or **14** (0.783 g, 2.29 mmol) were reacted with $NaBH_4$ (0.452 g, 11.73 mmol) in anhydrous methanol (27 mL), obtaining products **15** or **16** as a colourless oil. Yield: 0.325 g, 41%.

4.11.1. (1*R*,2*S*,4*S*,5*S*,7*R*)-4,5-diphenyl-1,11,11-trimethyl-3,6-diazatricyclo[6.2.1.0^{2,7}]undecane (15). IR (film): ν_{max}3062, 3031(νC^2_{sp}-H), 2927 (νC^3_{sp}-H), 1491 (νC^3_{sp}-N), 1452 (δC^3_{sp}-H) cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 0.88 (s, 3H; H-13 or H-14), 0.90 (s, 3H; H-13 or H-14), 1.03–1.10 (m, 2H; H-10), 1.48–1.59 (m, 2H; H-9), 1.69 (s, 3H; H-12), 1.78–1.90 (m, 3H; H-3, H-6, H-8), 2.88 (d, J_{7-2} = 7.3 Hz, 1H; H-2 or H-7), 3.16 (d, J_{2-7} = 7.3 Hz, 1H; H-2 or H-7), 3.60 (d, J_{4-5} = 8.4 Hz, 1H; H-4 or H-5), 4.06 (d, J_{4-5} = 8.4 Hz, 1H; H-4 or H-5), 7.21–7.23 (m, 2H; H-4′, H-4″), 7.26 (m, 4H; H-2′, H-6′, H-2″, H-6″), 7.25–7.27 (m, 4H; H-3′, H-5′, H-3″, H-5″) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ 11.4 (C-13 or C-14), 21.8 (C-12), 22.8 (C-13 or C-14), 27.5 (C-10), 35.7 (C-9), 47.4 (C-1 or C-11), 48.5 (C-1 or C-11), 50.8 (C-8), 58.3 (C-2 or C-7), 63.9 (C-4 or C-5), 65.9 (C-2 or C-7), 70.7 (C-4 or C-5), 126.8 (C-4′ or C-4″), 127.5 (C-4′ or C-4″), 127.6 (C-2′, C-2″ or C-6′, C-6″), 127.8 (C-2′, C-2″ or C-6′, C-6″), 128.3 (C-3′, C-3″ or C-5′, C-5″), 128.6 (C-3′, C-3″ or C-5′, C-5″), 144.2 (C-1′ or C-1″), 144.9 (C-1′ or C-1″) ppm. MS (EI, 70 eV): *m*/*z* (%) = 347 (27) [M+H$^+$], 346 (100) [M], 342 (32) [M–4H$^+$]. Anal. Calcd for $C_{24}H_{30}N_2$ (346.5): C, 83.19; H, 8.73; N, 8.08. Found: C, 83.27; H, 8.80; N, 8.10.

4.11.2. (1*R*,2*S*,4*R*,5*R*,7*R*)-4,5-diphenyl-1,11,11-trimethyl-3,6-diazatricyclo[6.2.1.0^{2,7}]undecane (16). IR (film): ν_{max} 3062, 3031 (νC^2_{sp} – H), 2927 (νC^3_{sp} – H), 1491 (νC^3_{sp} – N), 1452 (δC^3_{sp} – H) cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 0.86 (s, 3H; H-13 or H-14), 0.96 (s, 3H; H-13 or H-14), 1.05–1.21 (m, 2H; H-10), 1.52–1.74 (m, 2H; H-9), 1.65 (s, 3H; H-12), 1.75–1.80 (m, 1H; H-8), 1.95–2.10 (m, 1H; H-3 or H-6), 2.26–2.39 (m, 1H; H-3 or H-6), 2.97 (d, J_{7-2} = 7.2 Hz, 1H; H-2 o H-7), 3.02 (d, J_{2-7} = 7.3 Hz, 1H; H-2 or H-7), 3.65 (d, J_{4-5} = 7.9 Hz, 1H; H-4 or H-5), 4.18 (d, J_{5-4} = 7.9 Hz, 1H; H-4 or H-5), 7.16–7.24 (m, 2H; H-4′, H-4″), 7.25 (m, 4H; H-2′, H-6′, H-2″, H-6″), 7.26–7.27 (m, 4H; H-3′, H-5′, H-3″, H-5″) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ 12.1 (C-13 or C-14), 21.6 (C-12), 23.1 (C-13 or C-14), 27.0 (C-10), 36.4 (C-9), 47.5 (C-1 or C-11), 48.4 (C-1 or C-11), 50.6 (C-8), 61.5 (C-2 or C-7), 63.0 (C-4 or C-5), 65.9 (C-2 or C-7), 66.3 (C-4 or C-5), 126.8 (C-4′ or C-4″), 127.4 (C-4′ or C-4″), 127.5 (C-2′, C-2″ or C-6′, C-6″), 127.7 (C-2′, C-2″ or C-6′, C-6″), 128.4 (C-3′, C-3″ or C-5′, C-5″), 128.7 (C-3′, C-3″ or C-5′, C-5″), 144.5 (C-1′ or C-1″), 145.0 (C-1′ or C- cm^{-1} 1″) ppm. MS (EI, 70 eV): *m*/*z* (%) = 347 (33) [M+H$^+$], 346 (100) [M], 342 (32) [M–4H$^+$]. Anal. Calcd for $C_{24}H_{30}N_2$: C, 83.19; H, 8.73; N, 8.08. Found: C, 83.14; H, 8.68; N, 8.12.

4.12. Synthesis of 17 and 18

Compounds **15** and/or **16** were treated with 2 M HCl and the resulting hydrochlorides **17** and/or **18** were formed. The aqueous solution was concentrated to dryness in a dry-ice rotary evaporator, affording products **17** and/or **18** as yellowish solids in quantitative yield.

4.12.1. (1*R*,2*S*,4*S*,5*S*,7*R*)-4,5-diphenyl-1,11,11-trimethyl-3,6-diazatricyclo[6.2.1.0^{2,7}]undecane dihydrochloride (17). IR (KBr): ν_{max} 3348 (νN–H), 3041 (νC^2_{sp} – H), 2968 (νC^3_{sp} – H), 2958–2387 (νNH^+_2), 1570 (δN–H), 1451 (δC^3_{sp} – H) cm^{-1}. ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 0.84 (s, 3H; H-13 or H-14), 0.95 (s, 3H; H-13 or H-14), 1.15–1.30 (m, 2H; H-10), 1.23 (s, 3H; H-12), 1.58–1.72 (m, 1H; H-9a or H-9b), 1.78–1.90 (m, 1H; H-9a or H-9b), 2.02 (d, J_{8-9a} = 4.0 Hz, 1H; H-8), 3.82 (d, J_{2-7} = 8.4 Hz, 1H; H-2 or H-7), 3.92 (d, J_{2-7} = 8.4 Hz, 1H; H-2 or H-7), 5.00 (d, J_{4-5} = 11.6 Hz, 1H; H-4 or H-5), 5.04 (d, J_{4-5} = 11.2 Hz, 1H; H-4 or H-5), 7.20–7.22 (m, 2H; H-4′, H-4″), 7.24–7.26 (m, 4H; H-2′, H-6′, H-2″, H-6″), 7.29–7.34 (m, 4H; H-3′, H-5′, H-3″,

H-5″) ppm. ^{13}C NMR (100 MHz, D_2O, 25 °C): δ 10.6 (C-12), 20.5 (C-13 or C-14), 21.2 (C-13 or C-14), 26.3 (C-9), 34.5 (C-10), 48.1 (C-11), 48.8 (C-1), 49.0 (C-8), 56.0 (C-7), 59.5 (C-4 or C-5), 60.2 (C-4 or C-5), 66.7 (C-2), 128.5 (C-4′, C-4″), 128.8 (C-2′, C-2″ or C-6′, C-6″), 128.9 (C-2′, C-2″ or C-6′, C-6″), 129.6 (C-3′, C-3″ or C-5′, C-5″), 129.7 (C-3′, C-3″ or C-5′, C-5″), 142.3 (C-1′ or C-1″), 142.5 (C-1′ or C-1″) ppm. MS-FAB [FAB(+), NBA,]: *m/z* (%) = 349 (47) [(M–2HCl)+2H$^+$], 348 (71) [(M–2HCl)+H$^+$], 347 (100) [M–2HCl]. Anal. Calcd for $C_{24}H_{32}Cl_2N_2$: C, 68.73; H, 7.69; N, 6.68. Found: C, 68.65; H, 7.73; N, 6.61.

4.12.2. (1*R*,2*S*,4*R*,5*R*,7*R*)-4,5-diphenyl-1,11,11-trimethyl-3,6-diazatricyclo[6.2.1.0^{2,7}]undecane dihydrochloride (18). IR (KBr): ν_{max} 3348 (νN–H), 3041 ($\nu C^2_{sp}-H$), 2968 ($\nu C^3_{sp}-H$), 2958–2387 (νNH^+_2), 1570 (δN–H), 1451 ($\delta C^3_{sp}-H$) cm^{-1}. ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 0.86 (s, 3H; H-13 or H-14), 0.95 (s, 3H; H-13 or H-14), 1.15–1.30 (m, 2H; H-10), 1.26 (s, 3H; H-12), 1.58–1.72 (m, 1H; H-9a or H-9b), 1.78–1.90 (m, 1H; H-9a or H-9b), 1.98 (d, J_{8-9a} = 4.0 Hz, 1H; H-8), 3.82 (d, J_{2-7} = 8.4 Hz, 1H; H-2 or H-7), 3.92 (d, J_{2-7} = 8.4 Hz, 1H; H-2 or H-7), 4.83 (d, J_{4-5} = 6.4 Hz, 1H; H-4 or H-5), 4.87 (d, J_{4-5} = 6.0 Hz, 1H; H-4 or H-5), 7.20–7.22 (m, 2H; H-4′, H-4″), 7.24–7.26 (m, 4H; H-2′, H-6′, H-2″, H-6″), 7.29–7.34 (m, 4H; H-3′, H-5′, H-3″, H-5″) ppm. ^{13}C NMR (100 MHz, D_2O, 25 °C): δ 10.1 (C-12), 20.5 (C-13 or C-14), 21.3 (C-13 or C-14), 25.4 (C-9), 35.0 (C-10), 47.8 (C-11), 47.9 (C-1), 49.7 (C-8), 56.9 (C-7), 59.2 (C-4 or C-5), 59.8 (C-4 or C-5), 66.7 (C-2), 128.3 (C-4′, C-4″), 128.9 (C-2′, C-2″ or C-6′, C-6″), 129.4 (C-2′, C-2″ or C-6′, C-6″), 129.5 (C-3′, C-3″ or C-5′, C-5″), 129.8 (C-3′, C-3″ or C-5′, C-5″), 140.1 (C-1′ or C-1″), 140.8 (C-1′ or C-1″); MS-FAB [FAB(+), NBA,]: *m/z* (%) = 349 (47) [(M–2HCl)+2H$^+$], 348 (71) [(M–2HCl)+H$^+$], 347 (100) [M–2HCl]. Anal. Calcd for $C_{24}H_{32}Cl_2N_2$: C, 68.73; H, 7.69; N, 6.68. Found: C, 68.81; H, 7.71; N, 6.72.

4.13. Biological studies

4.13.1. Determination of the concentration of the compounds under evaluation. In order to express the concentration of the compounds with respect to the DNA base pairs, the molar ratio of compound to nucleotide (r_i) was used. This parameter reflects the proportion between the platinum complex and the base pairs of DNA (mol of compound/mol of nucleotide). The value of r_i was determined as follows:

$$r_i = \frac{m \cdot M_{nucl} \cdot A_m}{C \cdot M_r \cdot V}$$

where; m, mass of compound (μg); M_{nucl}, average formula weight per nucleotide (330 g/mol), A_m, number of metallic atoms per compound (for the complexes, we considered A_m = 1), C, DNA concentration (μg/mL), M_r, formula weight of each compound (g/mol) and V, volume of sample (mL).

4.13.2. Sample preparation for DNA interaction studies by circular dichroism. A stock solution of the platinum complex (1 mg mL^{-1}) in a TE [50 mM NaCl, 10 mM *tris*-(hydroxymethyl)aminomethane hydrochloride (Tris–HCl), 0.1 mM EDTA]:DMSO (98:2) mixture was prepared. The use of DMSO is to facilitate the dissolution of compounds to be evaluated. The pH of the solution was adjusted to 7.4 with 0.1 M NaOH (prepared with milli-Q water). A stock solution of CT DNA (*Calf Thymus DNA*) in TE was prepared (20 μg mL^{-1}) and kept at 4 °C before use. The final concentration of DNA was determined by measuring the absorbance at 260 nm in an UV–vis spectrophotometer. Drug-DNA complex formation was accomplished by addition of aliquots of the compound at different concentrations in TE buffer to the appropriate volume of the CT DNA solution (5 mL). The samples were prepared with an input molar ratio of the complex to nucleotide, r_i = 0.1, 0.3, 0.5. As a blank, a solution in TE of free native DNA was used. The reactions were run at 37 °C for 24 h in the dark.

4.13.3. Sample preparation for agarose gel electrophoresis. Stock solutions of the platinum complexes (1 mg mL^{-1}) in TE (50 mM NaCl, 10 mM Tris–HCl, 0.1 mM EDTA, pH 7.4): DMSO (98:2) mixture were prepared. Drug-DNA complex formation was accomplished by addition of the appropriate volume of the DNA pBR322 (0.25 mg/ mL) stock solution to aliquots of the compound at different concentrations in TE buffer. The samples were prepared with an input molar ratio of the complex to nucleotide, r_i = 0.1, 0.3, 0.5. The reactions were run at 37 °C for 24 h in the dark. Twenty microliters aliquots of complex-DNA compounds were submitted to 0.5% agarose gel electrophoresis for 5 h at 1.5 V/cm in 0.5XTBE (45 mM Tris–borate, 1 mM EDTA, pH = 8.0) buffer. The Gel was subsequently stained in the same buffer containing ethidium bromide (1 mg/mL) and photographed with a Fujifilm FTI-500 system.

4.13.4. Sample preparation for atomic force microscopy (AFM). An HEPES solution was prepared [40 mM of 4-(2-hydroxyethyl)-1-piperazinemethanesulfonic acid (HEPES), 10 mM $MgCl_2$, pH 7.4]. The DNA standard was prepared as follows: 1 μL of pBR322 DNA (Boehringer Mannheim GmbH) was dissolved in 39 μL of HEPES, the solution was heated at 60 °C for 30 min, to obtain a high number of OC forms, and incubated at 37 °C for 24 h in the dark. Stock solutions (1 mg mL^{-1}) of the complex in HEPES/DMSO (40:60) mixture were prepared. One microliter of DNA pBR322 (0.25 μg μL^{-1}) and an appropriate aliquot of the stock solution (r_i = 0.5) were diluted to 40 μL in HEPES. The samples were centrifuged for 2 min before incubation. The stock solutions of HEPES and of the complexes were filtered through a sterile 0.2 μm filter FP 030/3 to remove possible particles or aggregates in suspension. Finally, the samples were incubated at 37 °C for 24 h in the dark. The observation slides were prepared by placing a drop (6 μL) of DNA solution or DNA-complex solution onto a freshly cleaved mica disc (Ashville–Schoonmaker Mica Co., Newport New, V.A). After absorption of the sample on the mica surface for 5 min at room temperature, the discs were rinsed for 10 s in a jet of deionized water of 18 MΩ cm^{-1} from a Milli-Q water purification system (Millipore, Molshem,

France) directed onto the surface with a squeeze bottle. The samples were blow-dried with a flow of dry argon for 10 min before imaging by AFM.

4.13.5. Cytotoxicity against HL-60 cells. The growth inhibitory effect of platinum complexes on the leukaemia HL-60 cell line was measured by the MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The cell line HL60 (acute promyelocytic leukaemia, ATTCC No. CLL-240) was cultivated in RPMI-1640 supplemented with 2 mM L-glutamine and 10% FBS in sterile cultivation flasks into an incubator at 37 °C and under a controlled atmosphere (5% of CO_2 and 95% of humidity). To carry out the cell proliferation assays, the cells growing in the logarithmic phase were seeded in 96-well plates in a 100 μL volume of culture medium with a density of 10^4 cells/well. The cells were let to stand into the incubator for 2–3 h before performing any further treatment and then they were treated with varying doses of platinum complexes and the reference drug cisplatin at 37 °C for 24 h and 72 h. The treatment with the different complexes was carried out by adding 100 μL of medium to an aliquot of the standard solution of the complex in such a way that the final concentration in the well should be in between 0 and 200 μM. For each of the variants tested, four wells were used. Aliquots of 20 μL of MTT solution (per 200 μL of culture medium) were then added to each well. The plates were incubated a 37 °C and under 5% CO_2 for 2–3 h, depending on the metabolic capability of cells. After this time, the colour formed was quantified by a spectrophotometric plate reader (ELISA) at 495 nm wavelength, with a reference filter of 620 nm that allows the correction of background absorption produced by cell residues. These values of absorbance were also corrected by the average of the absorbance of blanks (having only culture medium). All experiments of cytotoxicity were independently performed in triplicate with quadruplicates of samples (4 wells per sample). The cell viability percentage was calculated by dividing the average absorbance of the cells treated with a platinum complex by that of the control. The IC_{50} values (drug concentration at which 50% of the cells are viable relative to the control) were obtained by GraphPad Prism software, version 4.0.

The in vitro induction of apoptosis by platinum compounds was determined by a flow cytometry assay with Annexin V-FITC by using an Annexin V-FITC Apoptosis Detection Kit (Roche). Exponentially growing HL-60 cells in 6-well plates (7.5×10^5 cells/well) were exposed to concentrations equal to the IC_{50} of the platinum drugs for 24 h. After the cells were submitted to staining with the Annexin V-FITC and propidium iodide, the amount of apoptotic cells was analyzed by flow cytometry (BD FACSCalibur).

Acknowledgments

We thank the Spanish Ministry of Education and Science for financial assistance (BQU2002-00601, CTQ2005-01195/BQU, UB-ACIU-2007, CTQ2007-64843/BQU and BIO2004-05-879). The authors thank Dr. Francisca García (Cell Culture Facility) and Manuela Costa (Flow Cytometry Facility) for technical assistance.

References and notes

1. Rosenberg, B.; Van Camp, L.; Krigas, T. *Nature* **1965**, *205*, 698–699.
2. Rosenberg, B.; Van Camp, L.; Trosko, J. E.; Mansour, V. H. *Nature* **1969**, *222*, 385–386.
3. Piccart, M. J.; Lamb, H.; Vermorken, J. B. *Ann. Oncol.* **2001**, *12*, 1195–1203.
4. Schepman, M. A.; Veer, J. L.; Hartog, J. H.; Lohman, P. H.; Reedijk, J. *Biochemistry* **1985**, *24*, 707–713.
5. Sorenson, C. M.; Eastman, A. *Cancer Res.* **1988**, *48*, 4484–4488.
6. Reedijk, J. *Chem. Commun.* **1996**, 801–806.
7. Chu, G. *J. Biol. Chem.* **1994**, *269*, 787.
8. Fuertes, M. A.; Alonso, C.; Pérez, J. M. *Chem. Rev.* **2003**, *103*, 645–662.
9. Dolz, F.; Pinkerton, R. *Eur. J. Cancer* **1994**, *2*, 194–201.
10. Siddik, Z. H. *Cancer Chemother. Pharmacol.* **1988**, *21*, 19–24.
11. Bacha, D. M. *Cancer Treat. Rep.* **1986**, *70*, 865–869.
12. Kennedy, I. C. S. *Cancer Chemother. Pharmacol.* **1990**, *26*, 232–234.
13. Andre, T.; Bensmaine, M. A.; Louvet, C.; Francois, E.; Lucas, V.; Desseigne, F. *J. Clin. Oncol.* **1999**, *17*, 3560–3568.
14. Diaz-Rubio, E.; Sastre, J.; Zaniboni, A.; Labianca, R.; Cortes-Funes, H.; de Braud, F. *Ann. Oncol.* **1998**, *9*, 105–108.
15. Extra, J. M.; Marty, M.; Brienza, S.; Misset, J. L. *Semin. Oncol.* **1998**, *25*, 13–22.
16. Ho, Y.; Au-Yeung, S. C.; To, K. K. *Med. Res. Rev.* **2003**, *23*, 633–655.
17. Wong, E.; Giandomenico, C. M. *Chem. Rev.* **1999**, *99*, 2451–2466.
18. Wheate, N. J.; Collins, J. G. *Coord. Chem. Rev.* **2003**, *241*, 133–145.
19. Pérez, J. M.; Fuertes, M. A.; Alonso, C.; Navarro-Raninger, C. *Crit. Rev. Oncol./Hematol.* **2000**, *35*, 109–120.
20. Rosell, R.; Abad, A.; Monzó, M.; Molina, F. in *Principios de la Quimioterapia Antineoplásica*, Ergón S. A., Madrid, **1995**.
21. (a) Gust, R.; Krauser, R.; Schmid, B.; Schönenberger, H. *Arch. Pharm. Med. Chem.* **1998**, *331*, 27; (b) Gust, R.; Schiinenberger, H.; Kritzenberger, J.; Range, K. J.; Klement, U.; Burgemeisterll, T. *Inorg. Chem.* **1993**, *32*, 5939.
22. (a) Chaney, S. G. *Int. J. Oncol.* **1995**, *6*, 1291–1305; (b) Boudny, V.; Vrana, O.; Gaucheron, F.; Kleinwachter, V.; Leng, M.; Brabec, V. *Nucleic Acids Res.* **1992**, *20*, 267; (c) Reedijk, J.; Lohman, P. H. M. *Pharmaceut. Weekbl. Sci. Ed.* **1985**, *7*, 173; (d) Page, J. D.; Hussain, I.; Sancar, A.; Chaney, S. G. *Biochemistry* **1990**, *29*, 1016; (e) Shellard, S. A. *Cancer Res.* **1991**, *51*, 4557; (f) Kidani, Y.; Inagaki, K. . *J. Med. Chem.* **1978**, *21*, 13–15; (g) Galansky, M.; Slaby, S.; Jakuper, M. A.; Keppeler, B. K. *J. Med. Chem.* **2003**, *46*, 4946; (h) Chaney, S. G.; Valsman, A. In *Platinum-Based Drugs in Cancer Therapy*; Kelland, L. R., Farrell, N. P., Eds.; Human Press: New Jersey, 2000; p 129; (i) Kido, Y.; Khokhar, A. R.; Al-Baker, S.; Siddik, Z. H. *Cancer Res.* **1993**, *53*, 4567; (j) Noji, M.; Okamoto, K.;

Kidani, Y. *J. Med. Chem.* **1981**, *24*, 508; (k) Siddik, Z. H.; al-Baker, S.; Thai, G.; Khokhar, A. R. *J. Cancer Res. Clin. Oncol.* **1994**, *120*, 409; (l) Witiak, D. T.; Rotella, D. P.; Filppi, J. A.; Gallucci, J. *J. Med. Chem.* **1987**, *30*, 1327; (m) Noji, M.; Okamoto, K.; Kidani, Y. *J. Med. Chem.* **1981**, *24*, 508.
23. Rentsch, K. M. *J. Biochem. Biophys. Methods* **2002**, *54*, 1.
24. (a) Ariens, E. J. *Eur. J. Clin. Pharmacol* **1984**, *26*, 663; (b) Ariens, E. J. In *Chirality in Drug Design and Synthesis*; Brown, C., Ed.; Academic Press: New York, 1990; p 29; (c) Lin, J. H.; Lu, A. Y. *Pharmacol. Rev.* **1997**, *49*, 403.
25. (a) Benedetti, M.; Malina, J.; Kasparkova, J.; Brabec, V.; Natile, G. *Environ. Health Perspect* **2002**, *110*, 779; (b) Coluccia, M.; Fanizzi, F. P.; Giannini, G.; Giordano, D.; Intini, F. P.; Lacidogna, G.; Loseto, F.; Mariggiò, M. A.; Nassi, A.; Natile, G. *Anticancer Res.* **1991**, *11*, 281; (c) Fanizzi, F. P.; Intini, F. P.; Maresca, F. L.; Natile, G.; Quaranata, R.; Coluccia, M.; Giordano, D.; Mariggio, M. A. *Inorg. Chim. Acta* **1987**, *137*, 45; (d) Misset, J. L. *Br. J. Cancer* **1998**, *77*, 4; (e) Spingler, B.; Whittington, D. A.; Lippard, S. J. *J. Inorg. Biochem.* **2001**, *86*, 440; (f) Spingler, B.; Whittington, D. A.; Lippard, S. J. *Inorg. Chem.* **2001**, *40*, 5596; (g) Vickery, K.; Bonin, A. M.; Fenton, R. R.; O'Mara, S.; Russell, P. J.; Webster, L. K.; Hambley, T. W. *J. Med. Chem.* **1993**, *36*, 3663.
26. (a) Rupe, H.; Bohny, P. *Hel. Chim. Acta* **1936**, *19*, 1305–1323; (b) Dornow, A.; Fust, K. J.; Jordan, H. D. *Chem. Ber.* **1957**, *90*, 2124–2137; (c) Busacca, C. A.; Campbell, S.; Dong, Y.; Grossbach, D.; Ridges, M.; Smith, L.; Spinelli, E. *J. Org. Chem.* **2000**, *65*, 4753–4755; (d) Nantz, M. H.; Lee, D. A.; Bender, D. M.; Roohi, A. H. *J. Org. Chem.* **1992**, *57*, 6653.
27. Pikul, S.; Corey, E. J.; Jeffrey, S. C.; White, J. D. *Org. Synth.* **1993**, *71*, 22–29.
28. Ivanov, V. I.; Minchenkova, L. E.; Schvolkina, A. K.; Poletavev, A. I. *Biopolymers* **1973**, *12*, 89–95.
29. Macquet, J. P.; Butour, J. L. *Biochemie* **1978**, *60*, 901–905.
30. Malinge, J. M.; Leng, M. *EMBO J.* **1984**, *3*, 1273.
31. Cohen, G. L.; Bauer, W. R.; Barton, J. K.; Lippard, S. J. *Science* **1979**, *203*, 1014–1016.
32. Bowler, B. E.; Hollis, S. L.; Lippard, S. J. *J. Am. Chem. Soc.* **1984**, *106*, 6102–6104.
33. Onoa, G. B.; Moreno, V. *Int. J. Pharm.* **2002**, *245*, 45–65.
34. Keck, M. V.; Lippard, S. J. *J. Am. Chem. Soc.* **1992**, *114*, 3386–3390.
35. Onoa, G. B.; Cervantes, G.; Moreno, V.; Prieto, M. J. *Nucleic Acids Res.* **1986**, *26*, 1473–1480.
36. Mosmann, T. *J. Immunol. Math.* **1983**, *65*, 55–63.
37. Tada, H. *J. Immunol. Math.* **1986**, *93*, 157–165.
38. Darzynkiewicz, Z.; Bruno, S.; Del Bino, G.; Gorczyca, W.; Hotz, M. A.; Lassota, P.; Traganos, F. *Cytometry* **1992**, *13*, 795–808.
39. Barinaga, M. *Science* **1993**, *259*, 762–763.
40. Bursch, W. *Biochem. Cell. Biol.* **1990**, *80*, 1071–1074.
41. Cotter, T. G. *Anticancer Res.* **1990**, *10*, 1153–1160.
42. Marx, J. *Science* **1993**, *259*, 760–761.
43. Vaux, D. L. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 786–789.
44. Williams, G. T. *Cell* **1991**, *65*, 1097–1098.