European Journal of Medicinal Chemistry 46 (2011) 1630–1637

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Design and synthesis of pironetin analogues with simplified structure and study of their interactions with microtubules

J. Alberto Marco [a,*], Jorge García-Pla [b], Miguel Carda [b], Juan Murga [b], Eva Falomir [b], Chiara Trigili [c], Sara Notararigo [c], J. Fernando Díaz [c,**], Isabel Barasoain [c,**]

[a] Depart. de Q. Orgánica, Univ. de Valencia, c/ D. Moliner 50, E-46100 Burjassot, Valencia, Spain
[b] Depart. de Q. Inorgánica y Orgánica, Univ. Jaume I, E-12071 Castellón, Spain
[c] CIB, Consejo Superior de Investigaciones Científicas, 28040 Madrid, Spain

ARTICLE INFO

Article history:
Received 22 October 2010
Received in revised form
29 January 2011
Accepted 8 February 2011
Available online 22 February 2011

Keywords:
Tubulin
Tubulin-active compound
Microtubule
Microtubule destabilization
Pironetin analogue
Cytotoxicity

ABSTRACT

The preparation of a series of pironetin analogues with simplified structure is described. Their cytotoxic activity and their interactions with tubulin have been investigated. It has been found that, while less active than the parent molecule, the pironetin analogues still share the mechanism of action of the latter and compete for the same binding site to α-tubulin. Variations in the configurations of their stereocenters do not translate into relevant differences between biological activities.

1. Introduction

Microtubules are dynamic polymers which play a central role in a number of cellular processes, most particularly cell division, as they are key constituents of the mitotic spindle [1]. Their shape can be described as hollow tubes of about 25 nm external diameter constituted of a protein named tubulin. The functional form of this protein is a heterodimer formed in turn through non-covalent binding of two monomeric constituents. These are two very similar polypeptides of about 450 amino acid residues which are called α- and β-tubulin [2]. For cell division to occur in a normal way, microtubules must be in a constant state of formation and disruption, a process named microtubule dynamics in which GTP hydrolysis into GDP plays a key role [3].

It is easy to understand that any molecule which exerts some type of action on microtubule dynamics will be able to influence the cell division process, not only of normal cells but also of tumoral ones. Since such an influence may be exerted by molecules that bind to any of the tubulin components, it is not surprising that tubulin-binding molecules (TBM) constitute a most important class of anticancer agents [4]. TBM are able to interfere with microtubule assembly and functions, either by causing disruption of the microtubules or else through their stabilization. In both cases, this results in mitotic arrest of eukaryotic cells and subsequent cell death. Most of the hitherto described active drugs are natural products or derivatives thereof [5]. Major drugs can already be found on the market and many other promising compounds are in clinical trials [4,5].

TBM may be divided in two broad categories, those that bind to α-tubulin and those that bind to β-tubulin. The latter group is presently by far the most numerous and contains products which cause either disruption or stabilization of microtubules. Among the drugs that belong to this group, the venerable alkaloid colchicine [6] exerts its effects by causing disruption of microtubules. In contrast, another renowned representative of the same group, paclitaxel, was the first-described tubulin-interacting drug that was found to stabilize microtubules [7]. In spite of the fact that they exert opposite effects on the mitotic spindle, both drugs are known to bind to β-tubulin, even though to different sites within this protein. The mechanisms of action [8] of many of these TBM and the

* Corresponding author. Tel.: +34 96 3544337.
** Corresponding authors.
E-mail addresses: alberto.marco@uv.es (J.A. Marco), fer@cib.csic.es (J.F. Díaz), i.barasoain@cib.csic.es (I. Barasoain).

0223-5234/$ – see front matter © 2011 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2011.02.011

molecular aspects [9] of their interactions with tubulin have been studied using a broad palette of methods [10].

The number of products that bind to α-tubulin is very small, the naturally occurring 5,6-dihydro-α-pyrone pironetin (Fig. 1) being the first-reported example [11]. The compound is a potent inhibitor of tubulin assembly and has been found to arrest cell cycle progression in the G2/M phase [12]. This feature has motivated a number of groups to undertake total syntheses of this natural compound [13]. Some synthetic and biological studies on modified variants of pironetin have recently been published [14].

Some structure-activity (SAR) studies on pironetin have been reported [12]. These studies have shown that the presence of the conjugated C2–C3 double bond and of the hydroxyl group at C-9, either free or methylated, are essential for the biological activity. The presence of a (7*R*)-hydroxyl group also seems to be very relevant [12c]. The epoxidation of the C12–C13 double bond has been shown to cause a decrease in the activity [12a,b] but this may perhaps involve a deleterious effect of the oxirane ring, rather than the necessity of this C=C bond. No data are available about the importance of the remaining structural features [14]. It has been proposed that the Lys352 residue of the α-tubulin chain adds in a Michael fashion to the conjugated double bond of pironetin, therefore forming a covalent bond with C-3 of the pyrone ring (Fig. 1). In addition, it has been suggested that the Asn258 residue of α-tubulin holds the pironetin molecule through two hydrogen bonds to the pyrone carbonyl and the methoxyl oxygen atoms [12].

The appearance of resistances to existing drugs has led to a continuous need of developing new bioactive compounds that overcome such problems. Even though first observed in the case of antibiotics [15], resistances have also been reported to TBM [4c,e,h,16]. The investigation of new members of this compound class therefore constitutes an important goal in chemistry and pharmacology. As a member of the up to now small group of products that bind to α-tubulin, pironetin constitutes a pharmacologically interesting target. Thus, the purpose of our present research is the preparation of pironetin analogues that retain a substantial proportion of the biological activity of the natural metabolite while displaying a more simplified structure. Indeed, pironetin is not an extremely complex molecule but, with six sp^3 stereocenters, a total synthesis will be anyway lengthy enough as to make not too practical a preparation at large scale. Our investigation aims at establishing which elements of the pironetin molecule are essential for its activity and, desirably, at achieving an improvement of the latter.

2. Concept and design of analogues

In order to develop SAR studies based on the pironetin framework, we have considered a very simplified, starting structure where all elements that have not yet proven to be essential have been removed. In the initial phase of our research, the elements that have been maintained are, as commented above, the conjugated pyrone ring and the methoxy group at C-9. The hydroxyl group at C-7 has been removed in some substrates and retained in others, in order to see the degree of its influence on the activity. The configurations of the stereocenters have also been varied. All alkyl pendants (methyl, ethyl) and the isolated C12–C13 double bond have been removed. Accordingly, the selected target structures are shown in Chart 1. From these, compounds **1**–**4**, which lack the hydroxyl group at C-7, are all four possible stereoisomers with this constitution (two racemates, **1**/**3** and **2**/**4**). Likewise, structures **5**–**12**, where the C-7 hydroxyl is present, represent all eight possible stereoisomers with such a constitution (four racemates, **5**/**11**, **6**/**12**, **7**/**10** and **8**/**9**).

3. Synthetic work

The synthesis of pironetin analogues **1**–**4** (Chart 1) was performed according to the general concept depicted with detail in Scheme 1 for the case of compound **1**. Brown's asymmetric allylation [17] of *n*-hexanal afforded homoallyl alcohol **13** [17c,d]. The required chiral allylborane was prepared through reaction of allylmagnesium bromide with the commercially available (+)-diisopinocampheylboron chloride, (+)-Ipc$_2$BCl. Methylation of the free hydroxyl group of compound **13** yielded methyl ether **14** [18]. Ozonolytic cleavage of the olefinic bond in **14** was followed by Wadsworth-Emmons olefination of the intermediate aldehyde **15** under mild conditions [19] to yield the conjugate ester **16**. Hydrogenation of the olefinic bond and reduction of the ester function gave primary alcohol **18**, which was then oxidized to the corresponding aldehyde. The latter was then subjected to the same asymmetric allylation conditions as the starting *n*-hexanal to yield homoallyl **19** as a 86:14 mixture of diastereomers. Reaction of **19** with acryloyl chloride at low temperature gave acrylate **20**, which

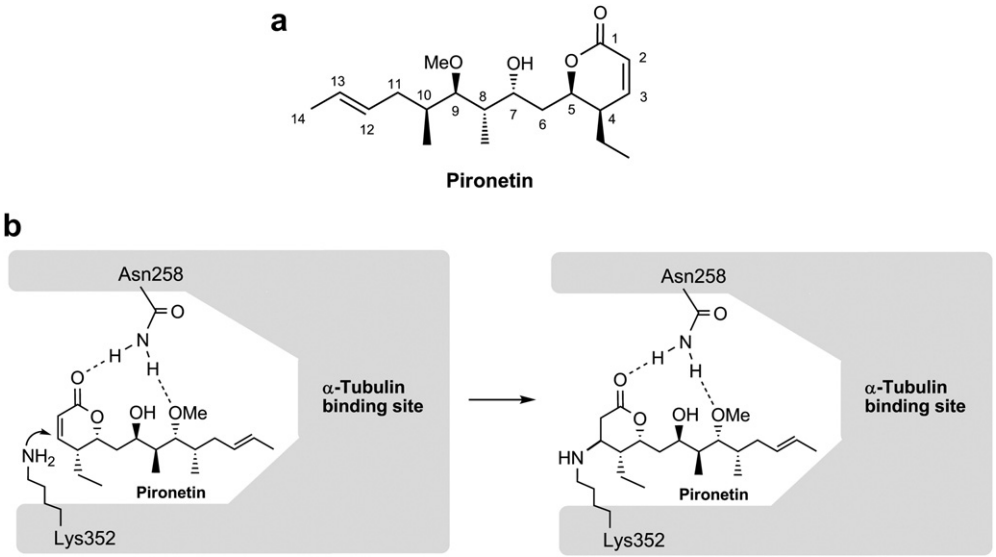


Fig. 1. (a) Structure of pironetin. (b) Schematic model of the covalent union of pironetin to its binding site at the α-tubulin surface.

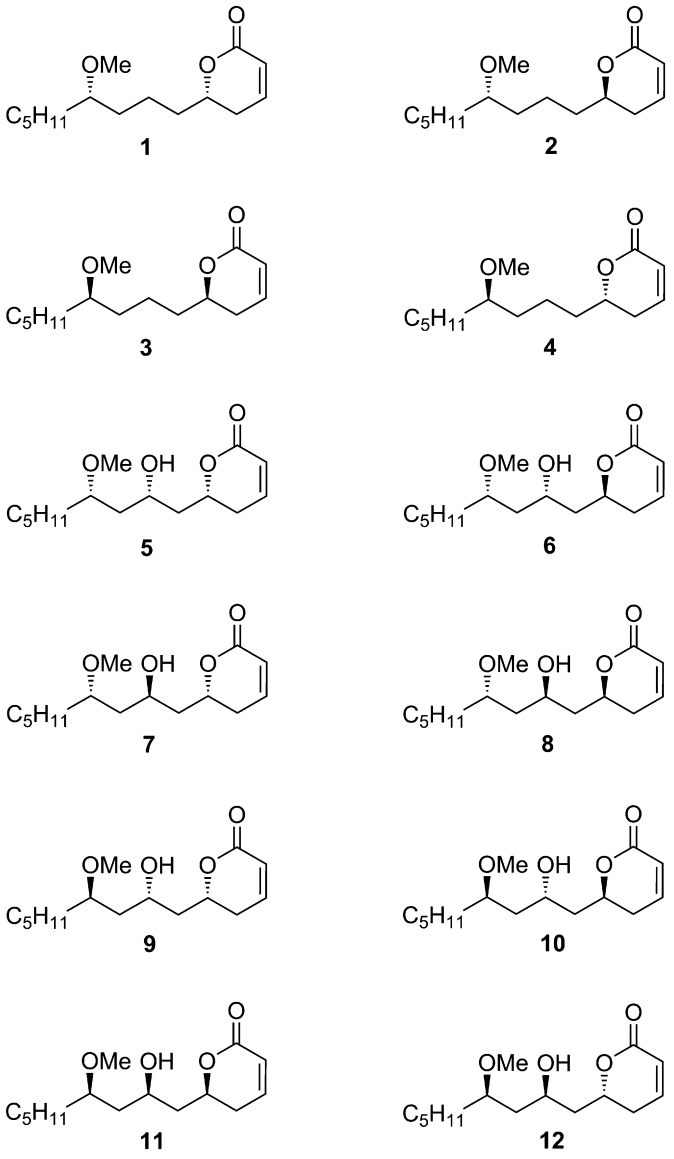


Chart 1. Structures of simplified pironetin analogues.

was subjected to ring-closing metathesis (RCM) [20] in the presence of Grubbs first-generation catalyst **Ru-I**. This provided dihydropyrone **1** in high yield.

Pyrones **2**–**4** were prepared along the same sequence of reactions by using in each case the appropriate chiral allylborane, either (+)- or (−)-Ipc$_2$BCl. Yields and stereoisomeric ratios were very similar to those observed in **1** (for experimental details, see the Supporting information).

Pironetin analogues **5**–**12** (Chart 1) were prepared using the same synthetic concept. Scheme 2 illustrates this with detail in the case of pyrone **5**. Aldehyde **15** (Scheme 1) was subjected to asymmetric allylation with the chiral allylborane derived from (+)-Ipc$_2$BCl to yield homoallyl alcohol **21**, obtained as a 92:8 mixture of diastereoisomers. Silylation to **22** was followed by ozonolytic cleavage of the C=C bond. Without purification, the intermediate aldehyde was submitted to asymmetric allylation using again the same chiral allylborane as before. This provided homoallyl alcohol **23** as a single diastereoisomer. This indicates that the minor stereoisomer was removed after this step, either during the chromatographic separation or via a kinetic resolution in the reaction. Reaction of **23** with acryloyl chloride at low temperature gave acrylate **24**, which was subjected to RCM in the presence of Grubbs first-generation catalyst **Ru-I** to furnish dihydropyrone **25** in good yield. Acid-catalyzed cleavage of the silyl group afforded pironetin analogue **5**.

Pyrones **6**–**12** were prepared along the same sequence of reactions by using in each case the appropriate chiral allylborane, either (+)- or (−)-Ipc$_2$BCl. Yields and stereoisomeric ratios were very similar to those observed in **5** (for experimental details, see the Supporting information).

After the synthesis of pironetin analogues **1**–**12** was completed, they were investigated in relation to their cytotoxic activity towards two types of tumoral lines and to their interactions as ligands with tubulin, always in comparison with the parental molecule, pironetin.

4. Results and discussion

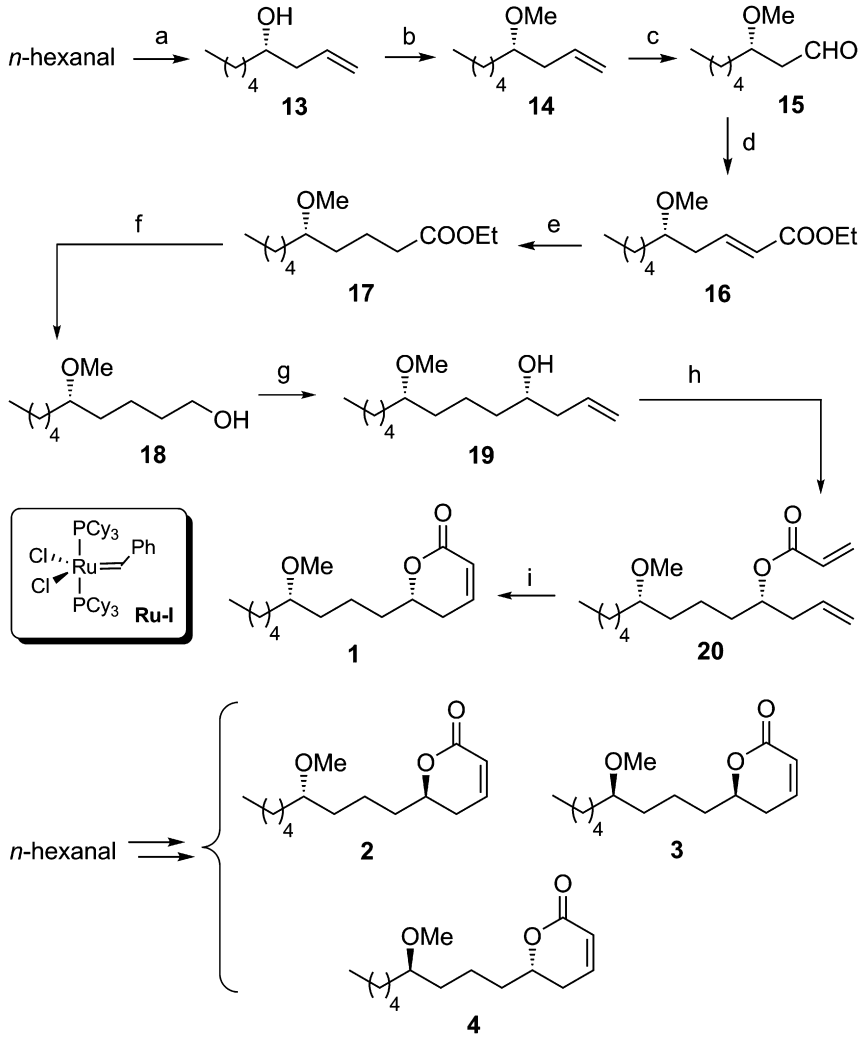
4.1. Cellular effects of the compounds

In order to check whether the newly synthesized compounds were cytotoxic through a mechanism similar to that of pironetin, we determined the IC_{50} values for all simplified analogs **1**–**12** and compared them with that of pironetin on A2780 and A2780AD human ovary carcinomas (Table 1). Pironetin was found active (IC_{50} 3 nM) in both the parental and the resistant cell lines. Furthermore, it has been found more cytotoxic than pironetin analogues **1**–**12** by 5–18 $\times$ 10^3 times in the case of A2780 cells and by 3–16 $\times$ 10^3 times in A2780AD cells. Indeed, and according to IC_{50} values, pironetin is active at concentrations in the nanomolar range whereas the pironetin analogues are active in the micromolar range. The latter killed both resistant and non resistant cells with a similar IC_{50} in the same way as pironetin, as expected for compounds with a covalent mechanism of action [21].

In order to establish whether compounds **1**–**12** act as microtubule depolymerizers in the same way as pironetin [12a,b], we studied the effect of these ligands on the microtubule cytoskeleton. We incubated cells in the presence of these ligands for 4 h (Fig. 2) or for 24 h (Figure A in the Supporting information). Pironetin at 50 nM concentration completely depleted cellular microtubules (C, D). In comparison, compounds **8** (E, F), **1** (G, H), **4** (I, J) and **3** (K, L) (Fig. 2) and **2** (Figure A in the Supporting information) were also active in microtubule depolymerization but at 200 μM concentration, i.e. 4 $\times$ 10^3 times less active than pironetin. In the preparations, we observed metaphase mitotic cells with type III mitotic spindles [22], with the DNA forming a ring surrounding a monoaster of microtubules, as with pironetin. With 24 h incubation and 200 μM concentration of **5**, **6**, **7**, **9**, **10**, **11** and **12** (Figure A in the Supporting information), the microtubule cytoskeleton was also disorganized and depolymerized as with compounds **1**, **2**, **3**, **4** and **8**. Aberrant mitosis was also present in all preparations.

We next studied whether ligands **1**–**12** were capable of blocking cells in the G2/M phase of the cell cycle of A549 (Fig. 3), as other microtubule modulating agents do. We incubated these cells for 20 h in the presence of the different ligands (**1**–**12**) or the drug vehicle. Pironetin at 50 nM concentration almost completely arrested cells in the G2/M phase. In the case of compounds **1**–**12**, an increase in the number of cells in this phase with respect to the control cells was observed. This effect was more pronounced with compounds **5**–**12**, the ligands where the C-7 hydroxyl is present (Fig. 3). At higher concentrations of the ligands, however, we did not see an increase in the number of cells arrested in the G2/M phase but a great cytotoxic effect.

We now conclude that, even if these pironetin analogues are less cytotoxic than pironetin itself, they also perturb the microtubular



Scheme 1. Synthesis of pironetin analogues **1**–**4**. (a) (+)-Ipc$_2$BCl, allylMgBr, Et$_2$O, −78 °C, 1 h, then addition of *n*-hexanal, 1 h, −78 °C, 95% (e.r. 91:9); (b) NaH, THF, 0 °C, then MeI, RT, overnight; (c) O_3, CH_2Cl_2, −78 °C, then PPh_3; (d) LiCl, *i*Pr$_2$NEt, (EtO)$_2$P(O)CH$_2$CO$_2$Et, MeCN, RT, overnight (60% overall from **13**); (e) H_2, Pd/C, EtOH, RT (quant.); (f) DIBAL, hexane, −78 °C–0 °C (98%); (g) Swern oxidation, then (+)-Ipc$_2$BCl, allylMgBr, Et$_2$O, −78 °C, 1 h, followed by addition of the aldehyde, 1 h, −78 °C (55% overall from **18**, d.r. 86:14); (h) CH_2=CHCOCl, CH_2Cl_2, *i*Pr$_2$NEt, −78 °C, 45 min (90%, d.r. 86:14); (i) 10% cat. **Ru-I**, CH_2Cl_2, Δ, 2 h (90%, d.r. 86:14). Acronyms and abbreviations: Ipc, isopinocampheyl; DIBAL, di*iso*butylaluminum hydride.

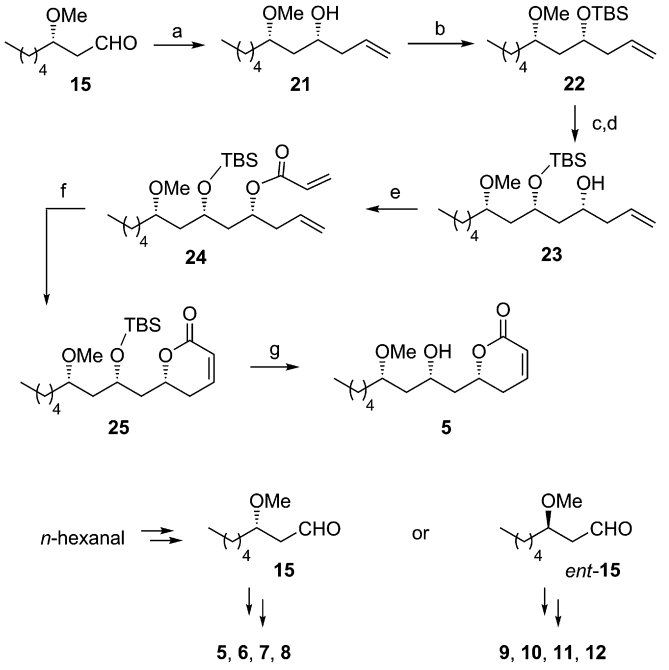
network. Analogues **1**, **2**, **5**, **6**, **7**, **8**, **9**, **10**, **11** and **12** accumulate cells in the G2/M phase but no apoptotic cells were observed, even with pironetin itself. Furthermore, they induce aberrant mitosis and give rise to a depolymerized microtubule cytoskeleton. Analogues **3** and **4** also induce aberrant mitosis and cause microtubule cytoskeleton depolymerization but are less effective in accumulating cells in the G2/M phase of the cell cycle.

4.2. Inhibition of microtubule assembly by pironetin analogues *1*–*12*

The observation of a weak microtubule assembly inhibition activity in cells by compounds **1**–**12** led us to study their ability to inhibit purified tubulin microtubule assembly. This aims at ascertaining whether the observed activity is exerted by means of tubulin-binding in a pironetin-like way. With this purpose in mind, the critical concentration [23] required for tubulin assembly was determined in GAB in the presence of a large excess (100 μM) of pironetin analogues **1**–**12**. Indeed, the concentration of tubulin required to produce assembly raises from 3.30 ± 0.10 μM in the absence of the pironetin analogues (DMSO vehicle) to a maximum value of 3.85 ± 0.16 μM in their presence. The highest activity was shown by compounds **1**–**4** lacking the hydroxyl group at C-7 (Cr for **1**, 3.73 ± 0.35 μM; Cr for **2**, 3.75 ± 0.11 μM; Cr for **3**, 3.79 ± 0.10 μM; Cr for **4**, 3.85 ± 0.16 μM), whereas the weakest compounds were **9** (Cr, 3.39 ± 0.07 μM) and **12** (Cr, 3.44 ± 0.03 μM). For the sake of comparison, values for docetaxel (a microtubule-stabilizing agent) and pironetin under these conditions are, respectively, 0.40 ± 0.12 μM and >15 μM. The values measured for analogues **1**–**12** confirm that the observed effect in cells is due to their interaction with tubulin. Moreover, the observed in vitro effect correlates well with the measured cytotoxicities.

4.3. Competition between pironetin and analogues as to their binding ability to tubulin

When a ligand does not show a visible change in a measurable property (e.g. absorbance or fluorescence) upon binding to a protein, the accurate characterization of such an interaction may become a complex task. In those cases it is possible to observe the existence of the aforementioned interaction by means of competition of the ligand under study with a reference ligand for the same binding site. When such a competition actually takes place, the



Scheme 2. Synthesis of pironetin analogues **5**–**12**. (a) (+)-Ipc$_2$BCl, allylMgBr, Et$_2$O, −78 °C, 1 h, then addition of aldehyde, 1 h, −78 °C (d.r. 92:8); (b) TBSOTf, CH_2Cl_2, 2,6-lutidine, 0 °C, 1 h (75% from **15**); (c) O_3, CH_2Cl_2, −78 °C, then PPh_3; (d) (+)-Ipc$_2$BCl, allylMgBr, Et$_2$O, −78 °C, 1 h, followed by addition of the aldehyde, 1 h, −78 °C (90%, d.r. > 95:5); (e) CH_2=CHCOCl, CH_2Cl_2, *i*Pr$_2$NEt, −78 °C, 45 min (90%); (f) 10% cat. **Ru-I**, CH_2Cl_2, Δ, 2 h (86%); (g) PPTS (cat.), MeOH, Δ, overnight (69%). Acronyms and abbreviations: TBS, *tert*-butyldimethylsilyl; Tf, trifluoromethanesulfonyl; PPTS, pyridinium *p*-toluenesulfonate.

attachment of the reference ligand to the binding site becomes perturbed by the presence of other ligands, whereby it may be presumed that they are competing for the same binding site [24]. This is a widely used technique in many situations, including the case of tubulin, which exhibits multiple binding sites, both activating and deactivating ones [25–28].

In the present investigation, the ligands under study are pironetin analogues **1**–**12**, whereas pironetin, a well-characterized α-tubulin ligand [12d], is the reference ligand. In order to confirm that compounds **1**–**12** share with the parent molecule the same binding site in tubulin, competition experiments between pironetin and some of its analogues were performed. As commented above, pironetin and, expectedly, also each one of ligands **1**–**12** become covalently attached to tubulin (Fig. 1). Assuming that a percentage of the pironetin binding sites is blocked by the ligand, it may be expected that a proportion of pironetin remains unreacted (i.e. not covalently bound) and can thus be measured by means of extraction with an organic solvent (for details, see Experimental, Section 6.2.4).

Fig. 4 shows that, in the absence of tubulin (Control), all pironetin (30 μM) remains unreacted in the liquid phase. In the presence of the vehicle (DMSO), 23.4 μM of pironetin is covalently bound to tubulin since only 6.6 μM can be extracted by the organic solvent. When the microtubules were previously incubated with the analogues, a significant percentage of the pironetin binding sites get blocked. As for the tubulin assembly inhibition, the compounds lacking the hydroxyl at C7 are the most powerful. In the presence of compounds **2**, **3** and **4**, nearly 10 μM of the pironetin binding sites are protected and approximately 15 μM pironetin remains unreacted. In the presence of compounds **6**, **9** and **10** approximately 4 μM of the pironetin binding sites remain protected and approximately 10 μM pironetin remains unreacted. Thus, concentrations of pironetin analogues **2**, **3**, **4**, **6**, **9** and **10** similar to those required to kill tumoral cells result in an increase of the critical concentration of purified tubulin required for the assembly, thus indicating that tubulin is actually their cellular target. The high concentrations of the analogues required to block the pironetin binding site in α-tubulin indicate that they compete with pironetin for the same binding site, even though with a lower affinity.

Table 1
Cytotoxicity of pironetin analogues **1**–**12** in ovarian carcinoma cells sensitive (A2780) and resistant (A2780AD) to chemotherapy by P-glycoprotein overexpression[a].

Drug	A2780 (μM)[b]	A2780AD (μM)	R/S[c]
Taxol	0.00082 ± 0.0002	0.949 ± 0.38	1157
Pironetin	0.0029 ± 0.001	0.003 ± 0.0002	1
1	20.6 ± 2.4	9 ± 3.2	0.45
2	14.9 ± 1.3	11.5 ± 1.3	0.77
3	14.4 ± 3.2	20.49 ± 5	1.42
4	17.1 ± 5	12.2 ± 1.9	0.7
5	53.7 ± 3	48 ± 0.01	0.89
6	35.4 ± 6.3	26.3 ± 4	0.74
7	33.7 ± 9	25.2 ± 0.86	0.74
8	49.4 ± 3.4	46.3 ± 6.8	0.93
9	44 ± 2.8	57.5 ± 0.35	1.3
10	22.9 ± 4.4	30 ± 1.3	1.3
11	54.5 ± 6	91.5 ± 23.6	1.67
12	28.9 ± 7	18.1 ± 3.2	0.62

[a] IC_{50} (50% inhibition of cell proliferation) of the ligands determined in ovarian carcinomas.
[b] IC_{50} values (μM) are the mean ± standard error of two independent experiments done in duplicate.
[c] The relative resistance of A2780AD cell line, obtained dividing the IC_{50} of the resistant cell line by the IC_{50} of the parental A2780 cell line.

5. Conclusion

As a general conclusion, it is worth highlighting that the synthesized pironetin analogues still retain a measurable proportion of the cytotoxicity of the natural product in spite of the deep-seated simplifying modifications performed on the structure of the natural product. Most importantly, they share the mechanism of action of the latter and compete with it for same binding site. From the various biochemical and biological data acquired, it seems that the configurations of the stereocenters do not seem to exert an outstanding influence on the cytotoxicity and on the ability of the compounds to bind to α-tubulin. Indeed, in the different assays performed the stereoisomeric analogues exhibit figures of the same order of magnitude. In contrast with that observed with pironetin itself, where the presence of a hydroxyl group at C-7 was found to be important [12], the removal of this hydroxyl group (compounds **1**–**4**) does not cause a suppression of the cytotoxicity or of the ability to depolymerize microtubules. However, these compounds are less effective in accumulating cells in the G2/M phase when compared with those having the C-7 hydroxy group. All these aspects will be taken into account when planning and designing the structures of the next generation of pironetin analogues. Reports in this sense will be published in due course.

6. Experimental

6.1. Chemistry. General procedures

The general reaction conditions and the physical and spectral data of all synthetic intermediates and final compounds are described in detail in the Supporting Information. The samples of compounds **1**–**12** used for the biological studies were purified to >95% by means of preparative HPLC.

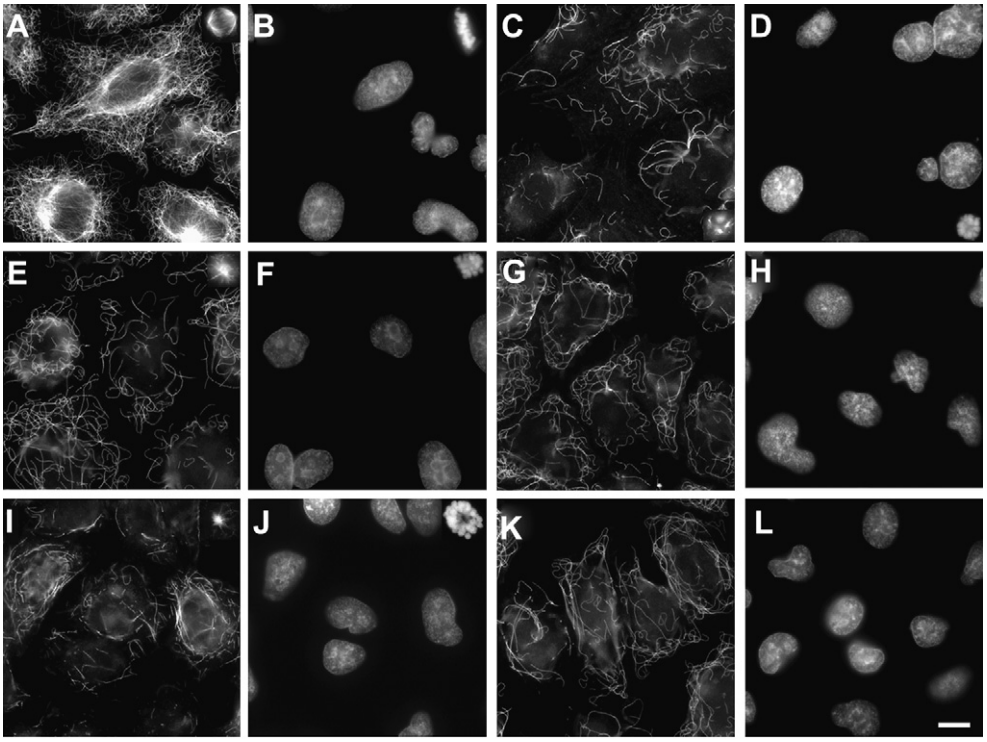


Fig. 2. Effect of pironetin analogues **1**, **3**, **4** and **8** as compared to the parent molecule pironetin on the microtubule network and on nucleus morphology. A549 cells were incubated for 4 h with either drug vehicle DMSO (A, B), 50 nM pironetin (C, D), 200 μM **8** (E, F), 200 μM **1** (G, H), 200 μM **4** (I, J) and 200 μM **3** (K, L). Microtubules are stained with α-tubulin antibodies (A, C, E, G, I, K) whereas DNA (B, D, F, H, J, L) was stained with Hoechst 33342. Insets (A, B, C, D, E, F, I, J) are mitotic spindles from the same preparation. The scale bar (L) represents 10 μm. All panels and insets have the same magnification.

6.2. Biological studies. Materials and methods

6.2.1. Cell culture

Human A549 non small lung carcinoma cells were cultured in RPMI 1640 supplemented with 10% FCS, glutamine, and antibiotics as previously described in Ref. [21]. Human ovarian carcinomas A2780 and A2780AD (MDR overexpressing P-glycoprotein) were cultured as above with the addition of 0.25 units/mL of bovine insulin.

6.2.2. Cytotoxicity assays, indirect immunofluorescence and cell cycle

Cytotoxic evaluation was performed with A2780 and A2780AD cells with the MTT assay modified as previously described in Ref.

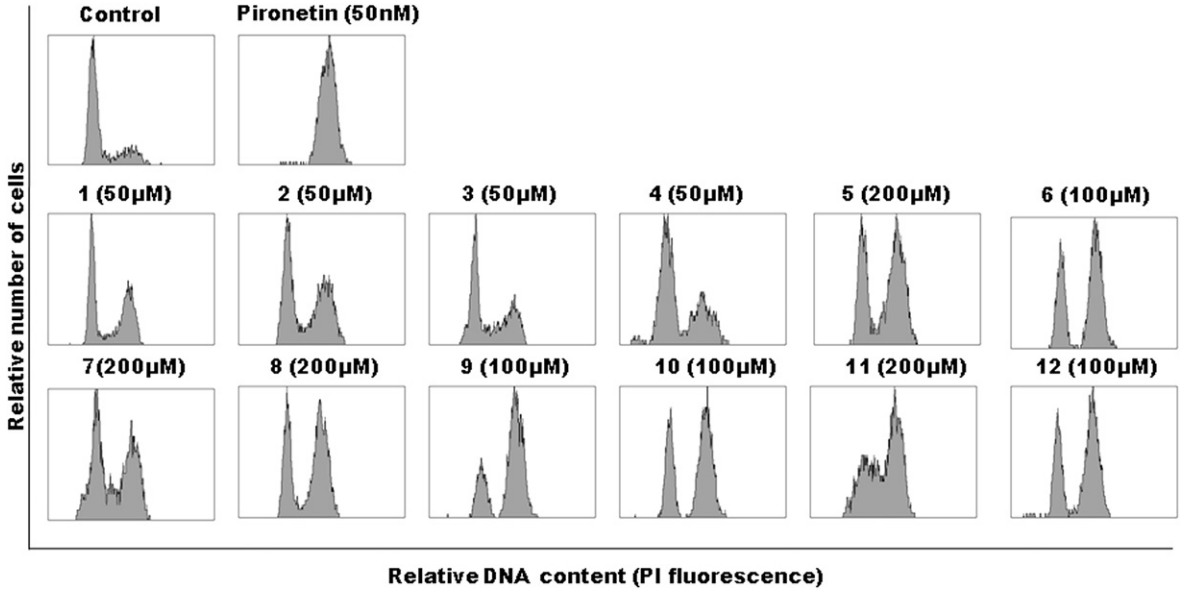


Fig. 3. Cell cycle histograms of A549 lung carcinoma cells untreated or treated with pironetin analogues **1**–**12**. The lowest ligand concentration that induces maximal arrest in the G2/M phase is depicted.

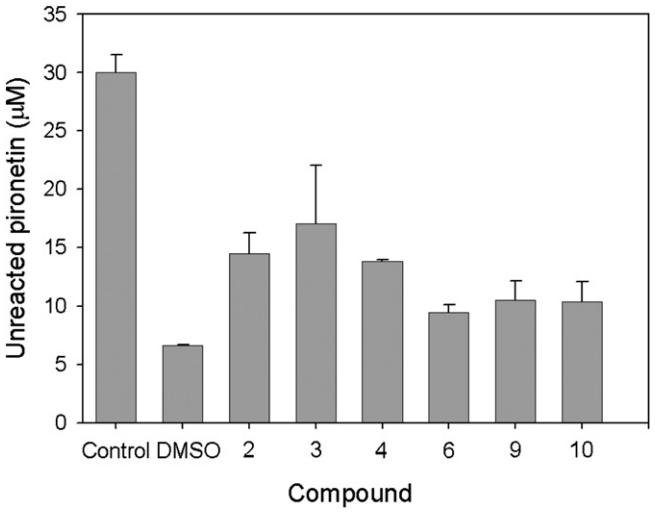


Fig. 4. Protection of the pironetin binding site by 200 μM concentrations of pironetin analogues **2**, **3**, **4**, **6**, **9** and **10**. Concentration of pironetin extracted with CH_2Cl_2 from the samples after 15 min of incubation of 30 μM pironetin with tubulin at 37 °C (control means the absence of tubulin in the buffer). Data represent average values of three measurements, errors are the standard errors of the average.

[29]. Indirect immunofluorescence was performed in A549 cells that had been cultured overnight in 12 mm round coverslips and incubated for further 24 h in the absence (drug vehicle DMSO) or in the presence of different ligand concentrations. Attached cells were permeabilized with Triton X100 and fixed with 3.7% formaldehyde. Microtubules were specifically stained with DM1A α-tubulin monoclonal antibodies and DNA with Hoechst 33342 as previously described in Ref. [30]. The preparations were examined using a Zeiss axioplan epifluorescence microscope and the images were recorded in a Hamamatsu 4742-95 cooled CCD camera. Progression through the cell cycle analysis was assessed by flow cytometry DNA determination with propidium iodide. Cells were fixed, treated with RNase and stained with propidium iodide as previously described in Ref. [31]. Analysis was performed with a Coulter Epics XL flow cytometer.

6.2.3. Tubulin assembly inhibition assay

The effect of the compounds in the assembly of purified tubulin was determined by incubating 20 μM purified tubulin at 37 °C for 30 min in GAB (glycerol assembling buffer, 3.4 M glycerol, 10 mM sodium phosphate, 1 mM EGTA, 1 mM GTP at pH 6.5) in the presence of 25 μM pironetin, docetaxel, 100 μM of each one of the analogs **1**–**12** or 2 μL DMSO (vehicle). The samples were processed and the critical concentration for tubulin assembly [23] in the presence of the ligands calculated as described [32]. The numerical data mentioned in the text (Section 4.2) represent average values of five measurements. Errors are standard errors of the average.

6.2.4. Competition between pironetin and the pironetin analogues for binding to tubulin

In order to check the binding site of the analogs in the tubulin dimer, 200 μL of 25 μM tubulin in GAB was incubated at 37 °C for 15 min in the presence of 200 μM of the respective pironetin analogue desired (compounds **2**, **3**, **4**, **6**, **9** and **10** were selected as representative examples) or of 10 μL of DMSO (vehicle). Subsequently, 30 μM pironetin was added and the reaction mixture was incubated for further 15 min, then 10 μM docetaxel was added to all samples as internal standard. After extraction of all samples three times with 1 volume of CH_2Cl_2, the extracts were dried and resuspended in 35 μL of 50% aqueous acetonitrile. The amount of non reacted pironetin was then determined by means of HPLC in an Agilent 1100 series instrument. Samples containing pironetin and compounds **6**, **9** and **10** were analyzed using a Zorbax Eclipse XDB-C18 column (4.6 × 150 mm, 5 micron). Elution was performed with a gradient of 50–80% acetonitrile in water at a flow rate of 1 mL/min for 30 min, with detection being followed through the UV absorbance at $\lambda = 230$ nm. Samples containing pironetin and compounds **2**, **3** and **4** were analyzed using two sequentially connected columns, a Zorbax Eclipse XDB-C18 (4.6 × 150 mm, 5 micron) column and a Supercosil LC18DB (4.6 × 250 mm, 5 micron) column. Elution was performed with a gradient of 60–80% acetonitrile in water at a flow rate of 1 mL/min for 30 min. As above, detection was followed through the UV absorbance at $\lambda = 230$ nm.

Acknowledgements

Financial support has been granted by the Spanish Ministry of Education and Science (CTQ2008-02800), by the Consellería d'Empresa, Universitat i Ciencia de la Generalitat Valenciana (ACOMP09/113) and by the BANCAJA-UJI Foundation (P1-1B2002-06 and P1-1B-2008-14). The biological work has been supported in part by grants from the Spanish Ministry of Education and Science (BIO2007-61336 and BIO2010-16351) and from the Comunidad de Madrid (BIPPED-CM) (both to J.F.D.). We further thank the Matadero Municipal Vicente de Lucas in Segovia for providing the calf brains which were the source of tubulin.

Appendix. Supplementary information

Supplementary data related to this article can be found online at doi:10.1016/j.ejmech.2011.02.011.

References

[1] The Role of Microtubules in Cell Biology, in: T. Fojo (Ed.), Neurobiology and Oncology, Humana Press, Totowa, New Jersey, 2008.
[2] (a) L.A. Amos, Org. Biomol. Chem. 2 (2004) 2153–2160;
(b) R.H. Wade, Mol. Biotechnol. 43 (2009) 177–191.
[3] (a) E. Nogales, H.-W. Wang, Curr. Opin. Cell Biol. 18 (2006) 179–184;
(b) E. Nogales, H.-W. Wang, Curr. Opin. Struct. Biol. 16 (2006) 221–229.
[4] (a) T. Beckers, S. Mahboobi, Drugs Future 28 (2003) 767–785;
(b) J.A. Hadfield, S. Ducki, N. Hirst, A.T. McGown, Prog. Cell Cycle Res. 5 (2003) 309–325;
(c) M.A. Jordan, L. Wilson, Nat. Rev. Cancer 4 (2004) 253–265;
(d) S.-H. Chen, J. Hong, Drugs Future 31 (2006) 123–150;
(e) E. Pasquier, M. Kavallaris, IUBMB Life 60 (2008) 165–170;
(f) P. Singh, K. Rathinasamy, R. Mohan, D. Panda, IUBMB Life 60 (2008) 368–375;
(g) P.G. Morris, M.N. Fornier, Clin. Cancer Res. 14 (2008) 7167–7172;
(h) E.A. Perez, Mol. Cancer Ther. 8 (2009) 2086–2095;
(i) S.M. Chen, L.-H. Meng, J. Ding, Expert Opin. Investig. Drugs 19 (2010) 329–343;
(j) D. Calligaris, P. Verdier-Pinard, F. Devred, C. Villard, D. Braguer, D. Lafitte, Cell. Mol. Life Sci. 67 (2010) 1089–1104.
[5] (a) K.-H. Altmann, J. Gertsch, Nat. Prod. Rep. 24 (2007) 327–357;
(b) D.G.I. Kingston, J. Org. Chem. 73 (2008) 3975–3984;
(c) D.G.I. Kingston, J. Nat. Prod. 72 (2009) 507–515.
[6] J. Chen, T. Liu, X. Dong, Y. Hu, Mini Rev. Med. Chem. 9 (2009) 1174–1190.
[7] Y. Fu, S. Li, Y. Zu, G. Yang, Z. Yang, M. Luo, S. Jiang, M. Wink, T. Efferth, Curr. Med. Chem. 16 (2009) 3966–3985.
[8] (a) M.A. Jordan, Curr. Med. Chem. – Anti-Cancer Agents 2 (2002) 1–17;
(b) M. Abal, J.M. Andreu, I. Barasoain, Curr. Cancer Drug Targets 3 (2003) 193–203.
[9] (a) J.J. Correia, S. Lobert, Curr. Pharm. Des. 7 (2001) 1213–1228;
(b) J. Jiménez-Barbero, F. Amat-Guerri, J.P. Snyder, Curr. Med. Chem. – Anti-Cancer Agents 2 (2002) 91–122;
(c) J.F. Díaz, J.M. Andreu, J. Jiménez-Barbero, Top. Curr. Chem. 286 (2009) 121–149;
(d) B. Gigant, A. Cormier, A. Dorléans, R.B.G. Ravelli, M. Knossow, Top. Curr. Chem. 286 (2009) 259–278;
(e) E.M. Daly, R.E. Taylor, Curr. Chem. Biol. 3 (2009) 367–379.
[10] (a) V.M. Sánchez-Pedregal, C. Griesinger, Top. Curr. Chem. 286 (2009) 151–208;
(b) J.H. Nettles, K.H. Downing, Top. Curr. Chem. 286 (2009) 209–257;

(c) M. Botta, S. Forli, M. Magnani, F. Manetti, Top. Curr. Chem. 286 (2009) 279–328.
[11] F. Sarabia, M. García-Castro, A. Sánchez-Ruiz, Curr. Bioact. Compd. 2 (2006) 269–299.
[12] (a) M. Kondoh, T. Usui, S. Kobayashi, K. Tsuchiya, K. Nishikawa, T. Nishikiori, T. Mayumi, H. Osada, Cancer Lett. 126 (1998) 29–32;
(b) M. Kondoh, T. Usui, T. Nishikiori, T. Mayumi, H. Osada, Biochem. J. 340 (1999) 411–416;
(c) H. Watanabe, H. Watanabe, T. Usui, M. Kondoh, H. Osada, T. Kitahara, J. Antibiot. 53 (2000) 540–545;
(d) T. Usui, H. Watanabe, H. Nakayama, Y. Tada, N. Kanoh, M. Kondoh, T. Asao, K. Takio, H. Watanabe, K. Nishikawa, T. Kitahara, H. Osada, Chem. Biol. 11 (2004) 799–806.
[13] (a) K. Yasui, Y. Tamura, K. Nakatani, K. Kawada, M. Ohtani, J. Org. Chem. 60 (1995) 7567–7574;
(b) M.K. Gurjar, J.T. Henri Jr., D.S. Bose, A.V.R. Rao, Tetrahedron Lett. 37 (1996) 6615–6618;
(c) N. Chida, M. Yoshinaga, T. Tobe, S. Ogawa, Chem. Comm. (1997) 1043–1044;
(d) H. Watanabe, H. Watanabe, M. Bando, M. Kido, T. Kitahara, Tetrahedron 55 (1999) 9755–9776;
(e) G.E. Keck, C.E. Knutson, S.A. Wiles, Org. Lett. 3 (2001) 707–710;
(f) L.C. Dias, L.G. de Oliveira, M.A. de Sousa, Org. Lett. 5 (2003) 265–268;
(g) X. Shen, A.S. Wasmuth, J. Zhao, C. Zhu, S.G. Nelson, J. Am. Chem. Soc. 128 (2006) 7438–7439;
(h) D. Enders, S. Dhulut, D. Steinbusch, A. Herrbach, Chem. Eur. J. 13 (2007) 3942–3949;
(i) C. Bressy, J.-P. Vors, S. Hillebrand, S. Arseniyadis, J. Cossy, Angew. Chem. Int. Ed. 47 (2008) 10137–10140;
(j) M.T. Crimmins, A.-M.R. Dechert, Org. Lett. 11 (2009) 1635–1638.
[14] (a) A. Vogt, P.A. McPherson, X.-Q. Shen, R. Balachandran, G.-Y. Zhu, B.S. Raccor, S.G. Nelson, M. Tsang, B.W. Day, Chem. Biol. Drug Des. 74 (2009) 358–368;
(b) J. Lin, X. Yue, P. Huang, D. Cui, F.-L. Qing, Synthesis (2010) 267–275.
[15] (a) L.M. Rossi, P. Rangasamy, J. Zhang, X.-Q. Qiu, G.Y. Wu, J. Pharm. Sci. 97 (2007) 1060–1070;
(b) P. Courvalin, J. Intern. Med. 264 (2008) 4–16;
(c) M. Gualtieri, F. Baneres-Roquet, P. Villain-Guillot, M. Pugniere, J.-P. Leonetti, Curr. Med. Chem. 16 (2009) 390–393.
[16] M. Kavallaris, Nat. Rev. Cancer 10 (2010) 194–204.
[17] (a) P.V. Ramachandran, G.-M. Chen, H.C. Brown, Tetrahedron Lett. 38 (1997) 2417–2420;
(b) P.V. Ramachandran, Aldrichimica Acta 35 (2002) 23–35;
(c) P.V. Ramachandran, B. Prabhudas, J.S. Chandra, M.V.R. Reddy, J. Org. Chem. 69 (2004) 6294–6304;
(d) For full spectral data of compounds 13 and ent-13, prepared via another procedure, see: M. Ishikawa, M. Amaike, M. Itoh, Y. Warita, T. Kitahara, Biosci. Biotechnol. Biochem. 67 (2003) 2210–2214.
[18] X. Li, J. Chen, X.-P. Cao, Synthesis (2006) 320–324.
[19] M.A. Blanchette, W. Choy, J.T. Davis, A.P. Essenfeld, S. Masamune, W.R. Roush, T. Sakai, Tetrahedron Lett. 25 (1984) 2183–2186.
[20] For reviews on ring-closing metathesis, see: (a) A. Fürstner, Angew. Chem. Int. Ed. 39 (2000) 3012–3043;
(b) L. Jafarpour, S.P. Nolan, Adv. Organomet. Chem. 46 (2000) 181–222;
(c) T.M. Trnka, R.H. Grubbs, Acc. Chem. Res. 34 (2001) 18–29;
(d) J.A. Love, in: R.H. Grubbs (Ed.), Handbook of Metathesis, vol. 2, Wiley-VCH, Weinheim, 2003, pp. 296–322;
(e) R.H. Grubbs, Tetrahedron 60 (2004) 7117–7140;
(f) D. Astruc, New J. Chem. 29 (2005) 42–56;
(g) A.H. Hoveyda, A.R. Zhugralin, Nature 450 (2007) 243–251.
[21] R.M. Buey, E. Calvo, I. Barasoain, O. Pineda, M.C. Edler, R. Matesanz, G. Cerezo, C.D. Vanderwal, B.W. Day, E.J. Sorensen, J.A. López, J.M. Andreu, E. Hamel, J.F. Díaz, Nat. Chem. Biol. 3 (2007) 117–125.
[22] M.A. Jordan, D. Thrower, L. Wilson, J. Cell Sci. 102 (1992) 401–416.
[23] F. Oosawa, S. Asakura, Thermodynamics of the Polymerization of Proteins. Academic Press, London, 1975.
[24] J.F. Díaz, R.M. Buey, in: J. Zhou (Ed.), Methods in Molecular Medicine, vol. 137, Humana Press Inc., Totowa, NJ, 2007, pp. 245–260.
[25] E. Hamel, Pharmacol. Ther. 52 (1992) 31–51.
[26] J.M. Andreu, B. Pérez-Ramírez, M.J. Gorbunoff, D. Ayala, S.N. Timasheff, Biochemistry 37 (1998) 8356–8368.
[27] D.E. Pryor, A. O'Brate, G. Bilcer, J.F. Díaz, Y.F. Wang, Y. Wang, M. Kabaki, M.K. Jung, J.M. Andreu, A.K. Ghosh, P. Giannakakou, E. Hamel, Biochemistry 41 (2002) 9109–9115.
[28] M.C. Edler, R.M. Buey, R. Gussio, A.I. Marcus, C.D. Vanderwal, E.J. Sorensen, J.F. Díaz, P. Giannakakou, E. Hamel, Biochemistry 44 (2005) 11525–11538.
[29] C. Yang, I. Barasoain, X. Li, R. Matesanz, R. Liu, F.J. Sharom, D.L. Yin, J.F. Díaz, W.S. Fang, ChemMedChem 2 (2007) 691–701.
[30] C. De Inés, D. Leynadier, I. Barasoain, V. Peyrot, P. Garcia, C. Briand, G.A. Rener, C. Temple Jr., Cancer Res. 54 (1994) 75–84.
[31] J.M. Andreu, I. Barasoain, Biochemistry 40 (2001) 11975–11984.
[32] R.M. Buey, I. Barasoain, E. Jackson, A. Meyer, P. Giannakakou, I. Paterson, S. Mooberry, J.M. Andreu, J.F. Díaz, Chem. Biol. 12 (2005) 1269–1279.