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Synthesis and Biological Evaluation of α-Tubulin-Binding Pironetin Analogues with Enhanced Lipophilicity

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Four new lipophilic analogues of the natural pyrone pironetin have been prepared. The C_9 side-chain of the latter has been replaced in one analogue by a 4-phenylbutyl chain and in the other three analogues by C_{13} or C_{16} aliphatic chains, all of them bearing two stereogenic centres. Their cytotoxic activities and interactions with tubulin have been investigated. It was found that all four are cytotoxic towards two either sensitive or resistant tumoral cell lines with similar IC_{50} values in each case, which indicates that, like the parent natural compound, they also display a covalent mechanism of action. However, one of them operates in all likelihood through a mechanism very similar to pironetin, whereas the other three seem to operate through a different mechanism.

Introduction

Microtubules are dynamic polymers that play a central role in a number of cellular processes, in particular, cell division, as they are key constituents of the mitotic spindle.[1] They can be described as hollow tubes with an external diameter of about 25 nm made of a protein named tubulin. The functional form of this protein is a heterodimer formed by the non-covalent binding of two monomeric species, namely two structurally related polypeptides of about 450 amino acid residues called α- and β-tubulin.[2] For cell division to occur in a normal way, microtubules must be in a constant state of assembly and disassembly, a process named microtubule dynamics in which the hydrolysis of GTP into GDP plays a key role.[3]

It is easy to understand why any molecule that 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 cells. Because such an influence may be exerted by molecules that bind to any of the tubulin components, it is not surprising that tubulin-binding molecules (TBMs) are a very important class of anticancer agents.[4] TBMs are able to interfere with microtubule assembly and functions, either by causing rupture of the microtubules or 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]

TBMs may be divided into 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 compounds that cause either rupture or stabilization of microtubules. Among the drugs that belong to this group, the well-known colchicine[6] exerts its effects by causing rupture to the microtubules. In contrast, another renowned representative of the same group, paclitaxel, was the first-described tubulin-interacting drug with the ability to stabilize microtubules.[7] Despite the fact that they exert opposite effects on the mitotic spindle, both drugs are known to bind to β-tubulin, although to different sites within the protein subunit. The mechanisms of action[8] of many of these TBMs and the molecular aspects[9] of their interactions with tubulin have been studied by a broad range of methods.[10]

The number of compounds reported to bind to α-tubulin is very small, the naturally occurring 5,6-dihydro-α-pyrone pironetin (Figure 1) being the first example,[11] followed a

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short time later by the peptide-like hemiasterlin family.[12] Pironetin proved a potent inhibitor of tubulin assembly and was found to arrest cell cycle progression in the G2/M phase.[13] This feature has motivated a number of groups to undertake total syntheses of this natural compound[14] and some synthetic and biological studies on modified variants of pironetin have previously been published.[15]

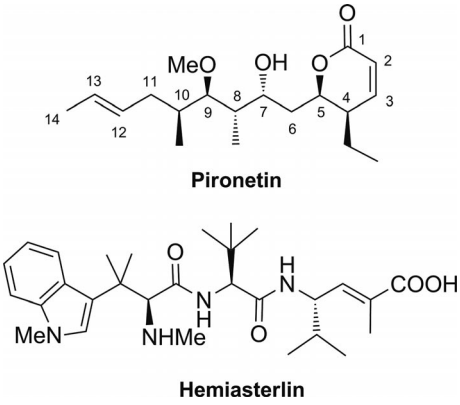


Figure 1. Structures of two natural products reported to selectively bind to α-tubulin.

Some structure–activity (SAR) studies on pironetin have also been reported.[13] These studies have shown that the presence of the conjugated C2–C3 double bond and of the hydroxy group at C-9, either free or methylated, are essential for biological activity. The presence of a (7*R*)-hydroxy group also seems to be very important.[13c] Epoxidation of the C12–C13 double bond has been shown to cause a decrease in activity,[13a,13b] but this may be a result of a deleterious effect of the oxirane ring, rather than a strict need for this C=C bond. No data are available on the importance of the remaining structural features.[15] It has been proposed that the Lys352 residue of the α-tubulin chain undergoes a Michael-type addition to the conjugated double bond of the pyrone ring of pironetin to form a covalent bond with C-3 (Figure 2). In addition, it has been suggested that the Asn258 residue of α-tubulin fixes the pironetin molecule through two hydrogen bonds with the pyrone carbonyl and the methoxy oxygen atom.[13]

The occurrence of resistance to existing drugs has led to a continuous need for the development of new bioactive compounds that overcome such problems. Although first observed for antibiotics, resistance has also been reported for TBMs.[4c,4e,4h,16] The synthesis of new members of this class of compounds therefore is an important goal in chemistry and pharmacology. As a member of the up-to-now small group of compounds that bind to α-tubulin, pironetin is a pharmacologically interesting target. Thus, the purpose of our current research is the preparation of pironetin analogues that retain a substantial proportion of the biological activity of the natural metabolite but that have a more simplified structure. Indeed, pironetin is not an extremely complex molecule but, with six sp^3 stereocentres, a total synthesis will be sufficiently lengthy that it is not very practical for its preparation on a large scale. Our investigation aims

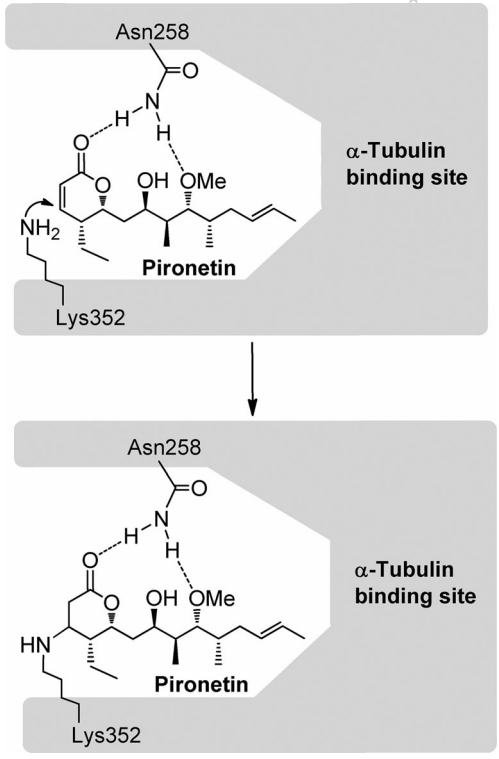


Figure 2. Schematic model of the covalent union of pironetin with its binding site at the α-tubulin surface.

at establishing which elements of the pironetin molecule are essential for its activity and, desirably, at improving this activity.

For SAR studies based upon the pironetin framework, we started by considering a simplified model structure in which all elements that have not yet proven to be essential were removed. The elements that remained were the conjugated pyrone ring and the side-chain with the methoxy group at C-9. The hydroxy group at C-7 was removed in some substrates and retained in others to investigate its influence on the activity. All alkyl pendants (methyls at C-8 and C-10, ethyl at C-4) and the isolated C12–C13 double bond were removed. The configurations of the three remaining stereocentres were varied systematically. Accordingly, the selected target structures are schematically shown in Figure 3. All four possible stereoisomers of **1**, with no hydroxy group at C-7, were prepared. In addition, all eight stereoisomers of **2**, with a hydroxy group at C-7, were synthesized.[17]

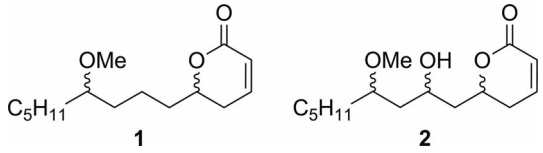


Figure 3. General structures of the simplified pironetin analogues **1** and **2**.[17]

The cytotoxic activities of these analogues and their interactions with tubulin were subsequently investigated. To measure the cytotoxic activities, ovarian carcinoma cells

sensitive (A2780) and resistant (A2780AD) to chemotherapy by P-glycoprotein overexpression were used.[17] It was found on the one hand that the analogues are cytotoxic in the low micromolar range, that is, they are about three orders of magnitude less active than the parent molecule.[17] On the other hand, they behaved in the same way as pironetin in that they killed both resistant and non-resistant cells with a similar IC_{50}, as expected for compounds with a covalent mechanism of action.[13] The general conclusion was that the synthetic pironetin analogues share the same mechanism of action as the natural compound and compete for the same binding site to α-tubulin, leading to rupture of the microtubule network. It is worth mentioning that variations in the configurations of the three stereocentres (C-5, C-7, C-9) does not translate into significant differences in biological activity.[17]

In continuation of our efforts in this line of research, we have now investigated the influence of the nature and size of the lipophilic side-chain attached to the dihydropyrone ring on the biological properties. It is our ongoing aim to prepare tubulin-active molecules with a hybrid structure and the ability to bind to two different points in the tubulin network. Because one half of these hybrid molecules will be pironetin-like, knowledge about the impact of the nature and size of the required spacer fragment is of paramount importance. In line with this reasoning, we have prepared the four pironetin analogues **3**–**6** (Figure 4). As regards the stereocentres, and in view of the aforementioned fact that their configurations do not seem to have a significant effect on their biological activity, compounds **3**–**6** were prepared with the same configuration as natural pironetin at C-5, C-7 and C-9. The difference between these compounds and the previously reported analogues **1** and **2** resides in the lipophilic end of the side-chain, which is much longer in **3** and **4**, compound **5** is an *O*-methylated analogue of **4** and compound **6** contains a phenyl ring instead of the aliphatic chain.

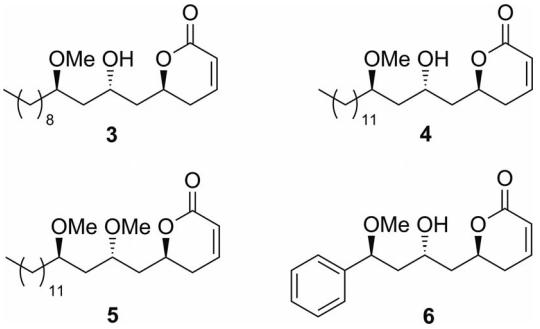
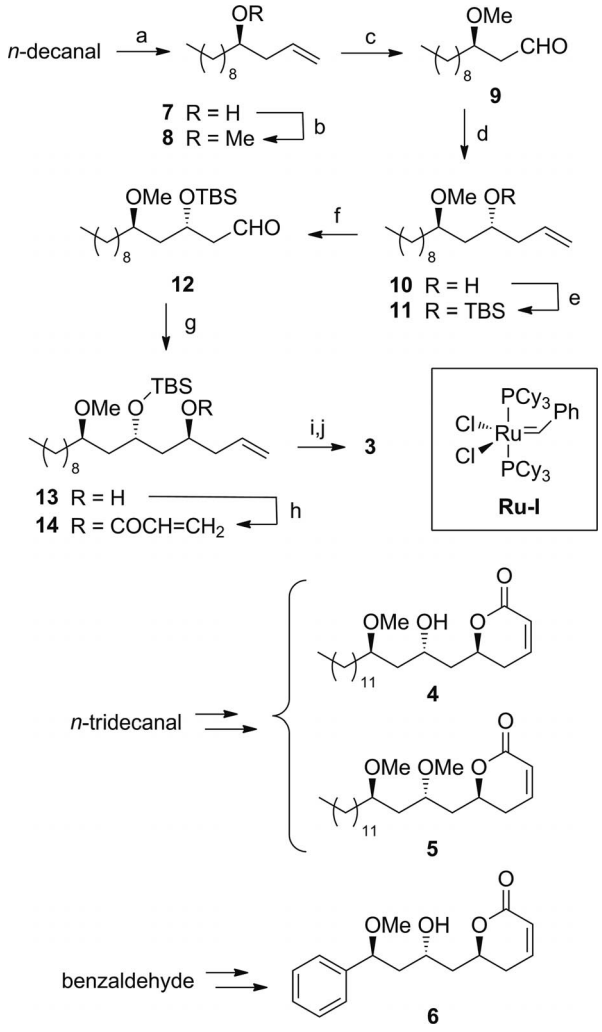


Figure 4. Structures of new pironetin analogues **3**–**6**.

Results and Discussion

Chemical Results

Dihydropyrones **3**–**6** were prepared by using the methodology employed for the synthesis of compounds of general structure **2**.[17] Scheme 1 shows the details of the synthetic route to pyrone **3** in which *n*-decanal was the starting material.



Scheme 1. Synthesis of pyrones **3**–**6**. Reagents and conditions: (a) (–)-Ipc_2BCl, allylMgBr, Et_2O, –78 °C, 1 h, then addition of *n*-decanal, 2 h, –78 °C, 95% (*er* 96:4); (b) NaH, THF, 0 °C, then MeI, r.t., overnight, 92%; (c) O_3, CH_2Cl_2, –78 °C, then PPh_3; (d) (+)-Ipc_2BCl, allylMgBr, Et_2O, –78 °C, 1 h, followed by addition of the aldehyde, 2 h, –78 °C, 70% overall from **8** (*dr* 88:12); (e) TBSOTf, CH_2Cl_2, 2,6-lutidine, room temp., 1 h, 90%; (f) O_3, CH_2Cl_2, –78 °C, then PPh_3; (g) (–)-Ipc_2BCl, allylMgBr, Et_2O, –78 °C, 1 h, followed by addition of the aldehyde, 2 h, –78 °C, 47% overall yield from **11** (*dr* > 95:5); (h) CH_2=CHCOCl, CH_2Cl_2, *i*Pr_2NEt, –78 °C, 45 min, 77%; (i) 1. 10% cat. Ru-I, CH_2Cl_2, Δ, 4 h, 85%; 2. PPTS (cat.), MeOH, Δ, overnight, 90%. TBS = *tert*-butyldimethylsilyl, PPTS = pyridinium *p*-toluenesulfonate, Ipc = isopinocampheyl, Tf = trifluoromethylsulfonyl.

Brown and co-workers' asymmetric allylation of *n*-decanal with a chiral allylborane afforded homoallyl alcohol **7**. The required borane was prepared by the reaction of allylmagnesium bromide with the commercially available (–)-diisopinocampheylboron chloride [(–)-Ipc_2BCl].[18] Methylation of the free hydroxy group of compound **7** yielded methyl ether **8**. Ozonolytic cleavage of the olefinic bond in **8** gave the intermediate aldehyde **9**, which was not isolated

but directly subjected to asymmetric allylation, again by using the chiral allylborane formed from allylmagnesium bromide and (+)-Ipc_2BCl. This gave rise to homoallyl alcohol **10** as an inseparable mixture of diastereoisomers in a ratio of 88:12. Silylation of this mixture gave **11**, which was subjected to ozonolytic cleavage of the C=C bond. Without purification, the intermediate aldehyde **12** was submitted to asymmetric allylation with the chiral allylborane generated from (–)-Ipc_2BCl and allylmagnesium bromide. This provided homoallylic alcohol **13** as a single diastereoisomer, the minor stereoisomer being removed during the chromatographic separation. Reaction of **13** with acryloyl chloride at low temperature gave acrylate **14**, which was then subjected to ring-closing metathesis[19] in the presence of Grubbs first-generation catalyst Ru-I. This afforded the corresponding dihydropyrone which, after acid-catalyzed desilylation, yielded pironetin analogue **3**.

An analogous reaction sequence starting from *n*-tridecanal served to prepare dihydropyrones **4** and **5**, whereas benzaldehyde was used for the synthesis of **6**. The complete details of these syntheses are given in the Supporting Information.

Biological Results

Cellular Effects of the Compounds

We determined the IC_{50} values for the action of analogues **3**–**6** on A2780 and A2780AD human ovary carcinomas and compared them with those of pironetin (Table 1). Pironetin proved active in both the parental and resistant cell lines, as expected for a compound with a covalent mechanism of action.[20] Although about three orders of magnitude less active than pironetin, analogues **3**–**6** were also found to be cytotoxic towards the A2780 and A2780AD cells and were able to kill both resistant and non-resistant cells with similar IC_{50} values.

Table 1. Effect of pironetin analogues **3**–**6** on the growth of A2780 and A2780AD (MDR overexpressing P-glycoprotein) ovarian carcinomas.[a]

Ligand	IC_{50} [μM][b]		R/S[c]
	A2780	A2780AD	
Pironetin	0.0062 ± 0.0012	0.0093 ± 0.0014	1.5
3	39.7 ± 0.5	38.6 ± 1.1	0.97
4	14.1 ± 0.424	12.5 ± 0.353	0.90
5	9.2 ± 0.3	10.9 ± 0.05	1.2
6	54.7 ± 5.4	48 ± 0.7	0.9

[a] IC_{50} (50% inhibition of cell proliferation) values for pironetin and compounds **3**–**6** in ovarian carcinomas. [b] IC_{50} values are given as the mean ± standard error of three independent experiments. [c] Resistance index (the relative resistance of A2780AD cell line, obtained by dividing the IC_{50} of the resistant cell line by that of the parental A2780 cell line).

To study the effect of compounds **3**–**6** on the microtubule cytoskeleton, we incubated cells in the presence of these compounds for 24 hours (Figure 5). Pironetin at a concentration of 50 nM completely depleted the cytoplasmic microtubules (Figure 5, C,D and inset): cells are arrested in the prometaphase[13a,13b] and type III mitotic spindles can be observed[21] with the chromosomes being arranged in a ball of condensed DNA enclosing one or more star-shaped aggregates of microtubules.

With 100 μM **3**, 25 μM **4** and 15 μM **5**, disorganization and some depolymerization of the microtubule cytoskeleton were observed: the cells become rounded and detach from the plastic substrate on which they are growing, with shrinking of the cell nucleus occurring in some cases (Figure 5, E–J). No mitotic cells were observed in these cell preparations. However, compound **6** at a concentration of 200 μM was found to cause microtubule depolymerization and cell arrest in the prometaphase, as in the case of pironetin (see Figure 5, K,L and inset).

We next studied whether compounds **3**–**6** were capable of blocking cells in the G2/M phase of the cell cycle of A549, as other microtubule modulating agents do. We incu-

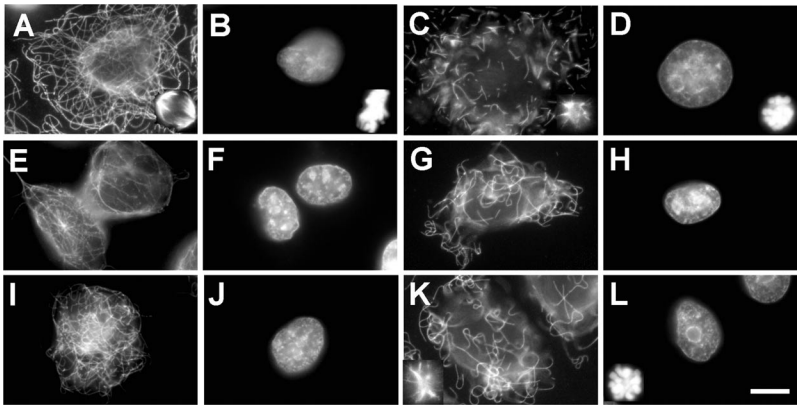


Figure 5. Effect of compounds **3**–**6**, in comparison with the parent molecule pironetin, on the microtubule network and nucleus morphology of A549 cells. Cells were incubated for 24 h with drug vehicle DMSO (A,B), 50 nM pironetin (C,D), 100 μM **3** (E,F), 25 μM **4** (G,H), 15 μM **5** (I,J) and 200 μM **6** (K,L). Microtubules were 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,K,L) are mitotic spindles of the same preparation. The scale bar in L represents 10 μm. All panels and insets have the same magnification.

bated these cells for 20 hours in the presence of the different compounds **3**–**6** or the drug vehicle (Figure 6). Pironetin at a concentration of 50 nM almost completely arrested the cells in the G2/M phase and, interestingly, so did **6** at a concentration of 200 μM. In contrast, compounds **3** (100 μM), **4** (50 μM) and **5** (10 μM) only caused a decrease in the number of G2/M cells with the appearance of subG1 cells, presumably dying cells. These results indicate that, although markedly less active, compound **6** is the only compound that behaves like pironetin. In addition, the results indicate that compounds **3**–**5**, although being active against the tubulin cytoskeleton, may exert their cytotoxicity through an alternative pathway.

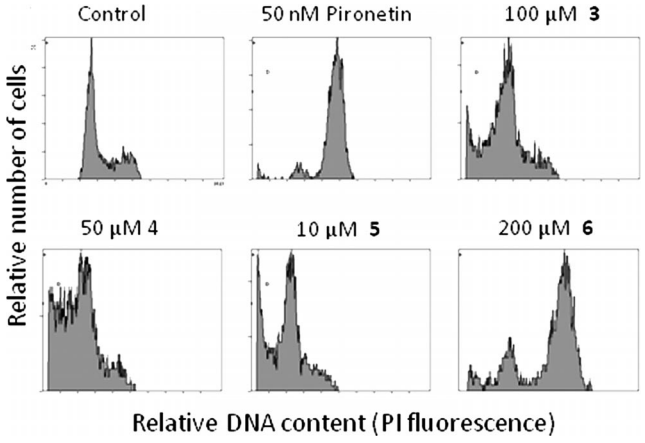


Figure 6. Cell cycle histograms of A549 lung carcinoma cells untreated and treated with pironetin and the pironetin analogues **3**–**6**. The lowest concentration that induces maximal effect on the cell cycle is depicted.

Tubulin Assembly

The critical concentration of tubulin required for assembly was determined in glycerol assembling buffer (GAB) in the presence of a large excess (100 μM) of compounds **3**–**6**. As shown in Table 2, the concentration of tubulin required to produce assembly (critical concentration[22]) oscillates between 3.3 μM in the absence of pironetin analogue and 4.9 μM in the presence of **5**, the most active of the compounds in this respect. The observed increase in the critical concentration required indicates that, as expected for a pironetin analogue, compounds **3**–**6** also inhibit the assembly of tubulin.

Table 2. Critical concentrationd (Cr) of tubulin required for microtubule assembly induced by pironetin analogues **3**–**6**.[a]

Ligand	Cr [μM][b]
Control	3.3 ± 0.3
Docetaxel	1.3 ± 0.4
3	3.8 ± 0.9
4	4.3 ± 1.3
5	4.9 ± 0.8
6	3.5 ± 0.8

[a] Concentrations are 25 μM for docetaxel and 100 μM for compounds **3**–**6**. [b] Cr values are the mean ± standard error of three independent experiments.

Conclusions

Four new pironetin derivatives **3**–**6** with an extended lipophilic side-chain have been synthesized with the aim of exploring the influence of the side-chain on their biological activities. All the compounds are cytotoxic in the micromolar range against both non-resistant and resistant P-glycoprotein overexpressing, multidrug ovarian carcinoma cell lines, similar IC_{50} values being found in both cell lines. However, although all the compounds are able to inhibit microtubule assembly, both in vitro and in cell cultures, thus sharing the general mechanism for the inhibition of action of tubulin assembly, compounds **3**–**5**, which contain a long aliphatic side-chain, differ from pironetin and compound **6** in that they do not accumulate cells in the G2/M phase of the cell cycle. This indicates that, in contrast to pironetin and **6**, compounds **3**–**5** trigger an alternative mechanism for cytotoxicity that leads to cell death.

Experimental Section

Chemical Procedures

General: ^1H and ^{13}C NMR spectra were recorded at 500 and 125 MHz in $CDCl_3$ solution at 25 °C. The signals of the deuteriated solvent ($CDCl_3$) were used as references (the singlet at δ = 7.25 ppm for ^1H NMR and the triplet centred at δ = 77.00 ppm for ^{13}C NMR). Carbon atom types (C, CH, CH_2, CH_3) were determined by using the DEPT pulse sequence. HRMS were recorded in electrospray mode (ESMS). IR data are given only for compounds with significant functions (OH, C=O) and were recorded as oily films on NaCl plates (oils) or as KBr pellets (solids). Optical rotations were measured at 25 °C. Reactions that required an inert atmosphere were carried out under N_2 in flame-dried glassware. Et_2O and THF were freshly distilled from sodium/benzophenone ketyl and transferred through a syringe. Dichloromethane was freshly distilled from CaH_2, tertiary amines were freshly distilled from KOH, and toluene was freshly distilled from sodium wire. Commercially available reagents were used as received. Unless detailed otherwise, "work-up" means pouring the reaction mixture into brine followed by extraction with the solvent indicated in parentheses. If the reaction medium was acidic, the mixture was also washed with 5% aq. $NaHCO_3$. If the reaction medium was basic, the mixture was also washed with aq. NH_4Cl. Further washing with brine, drying over anhydrous Na_2SO_4 and elimination of the solvent under reduced pressure were followed by chromatography on a silica gel column (60–200 μm) and elution with the solvent mixture indicated. When solutions were filtered through a Celite pad, the pad was additionally washed with the same solvent and the washings incorporated into the main organic layer.

(*R*)-Tridec-1-en-4-ol (7): Allylmagnesium bromide (commercial 1 M solution in Et_2O, 15 mL, 15 mmol) was added dropwise under N_2 through a syringe to a cooled solution (dry ice–acetone bath) of (–)-Ipc_2BCl (5.77 g, ca. 18 mmol) in dry Et_2O (75 mL). After the addition, the dry ice–acetone bath was replaced by an ice bath and the mixture was stirred for 1 h. The solution was allowed to stand, whereby precipitation of magnesium chloride took place. The supernatant solution was carefully transferred to another flask through a cannula. After cooling this flask to –78 °C, a solution of *n*-decanal (2.25 mL, 1.87 g, 12 mmol) in dry Et_2O (35 mL) was added dropwise through a syringe. The resulting solution was further stirred at –78 °C for 2 h. The reaction mixture was quenched

by the addition of a phosphate pH 7 buffer solution (15 mL), MeOH (15 mL) and 30% H_2O_2 (7 mL). After stirring for 30 min, the mixture was poured into satd. aq. $NaHCO_3$ and worked up (extraction with Et_2O). The residue was subjected to careful column chromatography on silica gel (hexanes, then hexanes/EtOAc, 9:1) to afford **7** (2.26 g, 95%) as an oil. $[\alpha]_D$ = +3.3 (*c* = 1.1, $CHCl_3$). The physical and spectroscopic data are as reported.[23] The enantiomeric ratio was found to be 96:4 by means of chiral HPLC using a Kromasil 5-AmyCoat column (4.6 × 20 mm). Elution was performed with hexane/2-propanol (99:1) and a flux of 0.4 mL/min. Elution times of both enantiomers: 21.21 (*S*) and 22.87 min (*R*).

(*R*)-4-Methoxytridec-1-ene (8): Sodium hydride (60% slurry in mineral oil, amount equivalent to 20 mmol) under N_2 was washed twice with dry hexane and once with dry THF. THF (75 mL) was added and the suspension was cooled in an ice bath. Alcohol **7** (1.98 g, 10 mmol) was then dissolved in dry THF (25 mL) and added dropwise to the sodium hydride suspension. The mixture was then allowed to warm to room temperature. Subsequently, methyl iodide (1.87 mL, ca. 30 mmol) was added in one portion and the mixture was stirred overnight at room temp. Work-up (Et_2O) was followed by column chromatography on silica gel (hexanes/EtOAc, 19:1) to afford **8** (1.95 g, 92%) as an oil. $[\alpha]_D$ = +2.9 (*c* = 0.1, $CHCl_3$). IR and ^1H NMR spectroscopic data are as reported.[24] ^{13}C NMR (125 MHz): δ = 135.1, 80.6 (CH), 116.7, 37.8, 33.4, 31.9, 29.8, 29.7, 29.6, 29.4, 25.3, 22.7 (CH_2), 56.5, 14.1 (CH_3) ppm.

(4*R*,6*R*)-6-Methoxypentadec-1-en-4-ol (10): Olefin **8** (1.275 g, ca. 6 mmol) was dissolved in CH_2Cl_2 (100 mL) and cooled to –78 °C. A stream of ozone-containing air was then bubbled through the solution until complete consumption of the starting material (TLC monitoring). Ozone residues were then eliminated by bubbling a stream of N_2 through the mixture, which was then allowed to warm to room temperature, treated with PPh_3 (3.15 g, ca. 12 mmol) and stirred for 2 h. After removal of the solvent under reduced pressure, the crude residue was stirred for 10 min in cold pentane (40 mL) and filtered. The solution was then concentrated under reduced pressure and the crude residue containing **9** was used directly in the next step.

Allylmagnesium bromide (commercial 1 M solution in Et_2O, 8 mL, 8 mmol) was added dropwise under N_2 through a syringe to a cooled solution (dry ice–acetone bath) of (+)-Ipc_2BCl (3.2 g, ≈10 mmol) in dry Et_2O (50 mL). After finishing the addition, the dry ice–acetone bath was replaced by an ice bath and the mixture was stirred for 1 h. The solution was allowed to stand, whereby precipitation of magnesium chloride took place. The supernatant solution was carefully transferred into another flask through a cannula. After cooling this flask to –78 °C, a solution of the crude aldehyde **9** from above in dry Et_2O (15 mL) was added dropwise through a syringe. The resulting solution was stirred at –78 °C for a further 2 h. The reaction mixture was quenched by the addition of a phosphate pH 7 buffer solution (40 mL), MeOH (40 mL) and 30% H_2O_2 (20 mL). After stirring for 30 min, the mixture was poured into satd. aq. $NaHCO_3$ and worked up (extraction with Et_2O). The residue was subjected to careful column chromatography on silica gel (hexanes, then hexanes/EtOAc, 19:1) to afford **10** (1.077 g, 70% overall from **8**) as an oil containing an 88:12 mixture of diastereoisomers which were very difficult to separate and thus the mixture was used as such in the next step. For analytical purposes, an aliquot could be concentrated to about 95% purity by means of careful column chromatography. $[\alpha]_D$ = –22.8 (*c* = 0.8, $CHCl_3$). ^1H NMR (500 MHz): δ = 5.85 (ddt, *J* = 17, 10, 7 Hz, 1 H), 5.15–5.05 (br. m, 2 H), 3.95 (m, 1 H), 3.48 (m, 1 H), 3.37 (s, 3 H), 2.90 (br. s, 1 H, OH), 2.25 (m, 2 H), 1.70–1.40 (br. m, 4 H), 1.35–1.25 (br. m, 14 H), 0.89 (t, *J* = 7 Hz, 3 H) ppm. ^{13}C NMR (125 MHz): δ = 135.0, 79.3, 68.0 (CH), 117.5, 42.3, 39.0, 33.1, 31.9, 29.8, 29.6, 29.5, 29.3, 25.4, 22.7 (CH_2), 56.7, 14.1 (CH_3) ppm. HRMS (ES): calcd. for $C_{16}H_{32}NaO_2$ $[M + Na]^+$ 279.2300; found 279.2302.

(4*R*,6*R*)-4-(*tert*-Butyldimethylsilyloxy)-6-methoxypentadec-1-ene (11): Alcohol **10** (1.025 g, 4 mmol) was dissolved under N_2 in dry CH_2Cl_2 (20 mL) and treated sequentially with 2,6-lutidine (700 μL, 6 mmol) and TBSOTf (1.15 mL, 5 mmol). The reaction mixture was then stirred for 1 h at room temp. and worked up (extraction with CH_2Cl_2). Column chromatography on silica gel (hexanes/EtOAc, 19:1) afforded **11** (1.33 g, 90%) as an oil containing 88:12 mixture of diastereoisomers which were very difficult to separate and thus the mixture was used as such in the next step. For analytical purposes, an aliquot could be concentrated to about 95% purity by means of careful column chromatography. $[\alpha]_D$ = –8.2 (*c* = 0.1, $CHCl_3$). ^1H NMR (500 MHz): δ = 5.82 (ddt, *J* = 17, 10, 7 Hz, 1 H), 5.10–5.00 (br. m, 2 H), 3.93 (m, 1 H), 3.34 (m, 1 H), 3.30 (s, 3 H), 2.24 (m, 2 H), 1.60–1.40 (br. m, 4 H), 1.35–1.25 (br. m, 14 H), 0.90 (12 H, strong singlet of 9 H overlapping a methyl triplet at δ = 0.89 ppm), 0.08 (s, 6 H) ppm. ^{13}C NMR (125 MHz): δ = 18.1 (C), 134.9, 77.1, 68.6 (CH), 117.0, 42.8, 41.7, 33.0, 31.9, 29.9, 29.7, 29.6, 29.3, 24.8, 22.7 (CH_2), 55.7, 26.0 (×3), 14.1, –4.1, –4.7 (CH_3) ppm. HRMS (ES): calcd. for $C_{22}H_{46}NaO_2Si$ $[M + Na]^+$ 393.3165; found 393.3162.

(4*S*,6*R*,8*R*)-6-(*tert*-Butyldimethylsilyloxy)-8-methoxyheptadec-1-en-4-ol (13): Prepared in two steps from **11** (via the non-isolated aldehyde **12**) in 47% overall yield by using the same experimental conditions as in the synthesis of **7**. Careful chromatography on silica gel (hexane/Et_2O, 9:1, then 8:2) permitted the isolation of diastereomerically pure (by NMR) **13** as an oil. $[\alpha]_D$ = –4.1 (*c* = 0.1, $CHCl_3$). IR: $\tilde{\nu}_{max}$ = 3400 (br., OH) cm^{-1}. ^1H NMR (500 MHz): δ = 5.82 (ddt, *J* = 17, 10, 7 Hz, 1 H), 5.15–5.05 (br. m, 2 H), 4.18 (m, 1 H), 4.05 (m, 1 H), 3.50 (br. s, 1 H, OH), 3.29 (s, 3 H), 3.26 (m, 1 H), 2.30–2.10 (br. m, 2 H), 1.70–1.50 (br. m, 4 H), 1.35–1.25 (br. m, 16 H), 0.90 (12 H, strong singlet of 9 H overlapping a methyl triplet at δ = 0.89 ppm), 0.12 (s, 3 H), 0.10 (s, 3 H) ppm. ^{13}C NMR (125 MHz): δ = 17.9 (C), 135.0, 77.3, 69.3, 68.1 (CH), 117.0, 42.4, 42.1, 41.0, 32.8, 31.9, 29.9, 29.7, 29.6, 29.3, 24.7, 22.7 (CH_2), 55.6, 25.9 (×3), 14.1, –4.4, –4.9 (CH_3) ppm.

(4*S*,6*S*,8*R*)-6-(*tert*-Butyldimethylsilyloxy)-8-methoxyheptadec-1-en-4-yl acrylate (14): Compound **13** (415 mg, ca. 1 mmol) was dissolved under N_2 in dry CH_2Cl_2 (30 mL) cooled to –78 °C and treated sequentially with *N*,*N*-diisopropylethylamine (2.1 mL, 12 mmol) and acryloyl chloride (815 μL, ca. 10 mmol). The reaction mixture was stirred at –78 °C until consumption of the starting material was complete (about 45 min, TLC monitoring). Work-up (extraction with CH_2Cl_2) and column chromatography on silica gel (hexane/Et_2O, 9:1) provided **14** (361 mg, 77%) as an oil. $[\alpha]_D$ = +2.2 (*c* = 0.5, $CHCl_3$). IR: $\tilde{\nu}_{max}$ = 1727 (C=O) cm^{-1}. ^1H NMR (500 MHz): δ = 6.38 (dd, *J* = 17.3, 1.5 Hz, 1 H), 6.10 (dd, *J* = 17.3, 10.5 Hz, 1 H), 5.80 (dd, *J* = 10.5, 1.5 Hz, 1 H), 5.78 (ddt, *J* = 17, 10.2, 7 Hz, 1 H,), 5.10–5.00 (br. m, 3 H,), 3.86 (m, 1 H), 3.29 (s, 3 H), 3.28 (m, 1 H), 2.40 (m, 2 H), 1.85–1.40 (br. M, 6 H), 1.35–1.25 (14 H, br. m), 0.89 (12 H, strong singlet of 9 H overlapping a methyl triplet at δ = 0.89 ppm), 0.05 (s, 3 H), 0.04 (s, 3 H) ppm. ^{13}C NMR (125 MHz): δ = 165.7, 18.0 (C), 133.4, 129.0, 77.5, 71.2, 67.0 (CH), 130.2, 118.0, 42.7, 42.0, 39.0, 33.2, 31.9, 29.9, 29.7, 29.6, 29.3, 24.7, 22.7 (CH_2), 55.8, 26.0 (×3), 14.1, –4.3, –4.4 (CH_3) ppm. HRMS (ES): calcd. for $C_{27}H_{52}NaO_4Si$ $[M + Na]^+$ 491.3533; found 491.3529.

(6*S*)-6-[(2*S*,4*R*)-2-(*tert*-Butyldimethylsilyloxy)-4-methoxytridecyl]-5,6-dihydro-2*H*-pyran-2-one (15): Diolefin **14** (234 mg, ca. 0.5 mmol) was dissolved under N_2 in dry, degassed CH_2Cl_2 (50 mL) and treated with Grubbs first-generation catalyst [PhCH=$RuCl_2$-$(PCy_3)_2$] (41 mg, ca. 0.05 mmol). The mixture was heated at reflux until consumption of the starting material (ca. 4 h, TLC monitoring). Removal of the solvent under reduced pressure and column chromatography on silica gel (hexane/EtOAc, 19:1) yielded pyranone **15** (187 mg, 85%) as an oil. $[\alpha]_D$ = –6.5 (*c* = 0.2, $CHCl_3$). IR: $\tilde{\nu}_{max}$ = 1732 (C=O) cm^{-1}. ^1H NMR (500 MHz): δ = 6.86 (dt, *J* = 9.7, 4 Hz, 1 H), 6.00 (br. d, *J* = 9.7 Hz, 1 H), 4.58 (m, 1 H), 4.06 (m, 1 H), 3.28 (s, 3 H), 3.26 (quint., *J* ≈ 6 Hz, 1 H), 2.30 (m, 2 H), 2.00 (m, 1 H), 1.65–1.55 (br. m, 3 H), 1.55–1.40 (br. m, 2 H), 1.35–1.20 (br. m, 14 H), 0.87 (12 H, strong singlet of 9 H overlapping a methyl triplet at δ = 0.89 ppm), 0.07 (s, 3 H), 0.06 (s, 3 H) ppm. ^{13}C NMR (125 MHz): δ = 164.2, 18.0 (C), 145.1, 121.5, 77.7, 74.5, 66.3 (CH), 43.3, 43.1, 33.2, 31.9, 29.9, 29.7, 29.6, 29.5, 29.3, 24.7, 22.6 (CH_2), 55.8, 25.9 (×3), 14.1, –4.4, –4.5 (CH_3) ppm. HRMS (ES): calcd. for $C_{25}H_{48}NaO_4Si$ $[M + Na]^+$ 463.3219; found 463.3218.

(6*S*)-6-[(2*S*,4*R*)-2-Hydroxy-4-methoxytridecyl]-5,6-dihydro-2*H*-pyran-2-one (3): Compound **15** (132 mg, 0.3 mmol) was dissolved in MeOH (15 mL) and treated with PPTS (15 mg, 0.06 mmol) and water (0.15 mL). The mixture was then heated at reflux overnight, cooled and neutralized by addition of solid $NaHCO_3$. After filtering, the solution was evaporated under reduced pressure and the oily residue was subjected to column chromatography on silica gel (hexanes/EtOAc, 1:4) to yield **3** (88 mg, 90%) as an oil. $[\alpha]_D$ = –16 (*c* = 1, $CHCl_3$). IR: $\tilde{\nu}_{max}$ = 1712 (C=O) cm^{-1}. ^1H NMR (500 MHz): δ = 6.86 (m, 1 H), 6.00 (d, *J* = 9.5 Hz, 1 H), 4.72 (m, 1 H), 4.22 (m, 1 H), 3.45 (m, 1 H), 3.35 (br. s, 4 H, OMe + OH), 2.45–2.30 (br. m, 2 H), 1.90–1.40 (br. m, 6 H), 1.35–1.20 (br. m, 14 H), 0.87 (t, *J* = 6.8 Hz, 3 H) ppm. ^{13}C NMR (125 MHz): δ = 164.4 (C), 145.2, 121.4, 79.6, 75.1, 64.7 (CH), 42.9, 39.4, 32.8, 31.9, 30.0, 29.7, 29.6, 29.5, 29.3, 25.5, 22.6 (CH_2), 56.6, 14.1 (CH_3) ppm. HRMS (ES): calcd. for $C_{19}H_{34}NaO_4$ $[M + Na]^+$ 349.2354; found 349.2358.

Biological Procedures

Cell Culture: Human A549 non-small lung carcinoma cells were cultured in RPMI 1640 supplemented with 10% FCS, glutamine and antibiotics as described previously.[20] 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.

Cytotoxicity Assays, Indirect Immunofluorescence and Cell Cycle: Cytotoxic evaluation was performed with A2780 and A2780AD cells by using the MTT assay modified as described previously.[25] Indirect immunofluorescence was performed on A549 cells that had been cultured overnight in 12 mm round coverslips and incubated for a further 24 h in the absence (drug vehicle DMSO) or in the presence of different compound 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 described previously.[26] The preparations were examined with a Zeiss axioplan epifluorescence microscope and the images recorded by using a Hamamatsu 4742-95 cooled CCD camera. Progression of the cell cycle was analysed by flow cytometry DNA determination with propidium iodide. Cells were fixed, treated with RNase and stained with propidium iodide as described previously.[27] Analysis was performed with a Coulter Epics XL flow cytometer.

Tubulin Assembly Inhibition Assay: The effect of compounds **3**–**6** on 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 and 6 mM $MgCl_2$ at pH 6.5) in the presence of 25 μM docetaxel, 100 μM of one of the analogues **3**–**6** or 2 μL DMSO (drug vehicle). The samples were processed and the critical concentrations for tubulin assembly[22] in the presence of the compound were calculated as described previously.[27]

Supporting Information (see footnote on the first page of this article): Experimental procedures and details of the preparation of pyrones **4**–**6** and of all required synthetic intermediates, and ^1H and ^{13}C NMR spectra of all new compounds.

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