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Synthesis and Cytotoxicity Evaluation of C4- and C5-Modified Analogues of the α,β-Unsaturated Lactone of Pironetin

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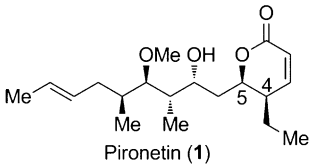
Pironetin is a natural product with potent antiproliferative activity that forms a covalent adduct with α-tubulin via conjugate addition into the natural product's α,β-unsaturated lactone. Although pironetin's α,β-unsaturated lactone is involved in its binding to tubulin, the structure–activity relationship at different positions of the lactone have not been thoroughly evaluated. For a systematic evaluation of the structure–activity relationships at the C4 and C5 positions of the α,β-unsaturated lactone of pironetin, twelve analogues of the natural product were prepared by total synthesis. Modifying the stereochemistry at the C4 and/or C5 positions of the α,β-unsaturated lactone of pironetin resulted in loss of antiproliferative activity in OVCAR5 ovarian cancer cells. While changing the C4 ethyl substituent with groups such as methyl, propyl, cyclopropyl, and isobutyl were tolerated, groups with larger steric properties such as an isopropyl and benzyl groups were not.

Introduction

Tubulin-binding anticancer agents act by disrupting microtubule dynamics during mitosis, which results in G2/M phase arrest, leading to apoptosis. Clinically used natural product-derived chemotherapeutics that disrupt tubulin dynamics include the taxanes, epothilone B, and vinca alkaloids,[1–3] as well as the antibody drug conjugates brentuximab vedotin and trastuzumab emtansine, which are based on the natural products dolastatin and maytansine.[4,5] X-ray crystallographic studies have shown that these natural products and other tubulin-binding natural products such as colchicine and the hemiasterlines bind to β-tubulin.[6–13] While these agents have been very successful for the treatment of a variety of cancers, drug-resistance to tubulin binding drugs has been associated with overexpression of P-glycoprotein and changes in the expression levels of β-tubulin isoforms.[14–16] Given the success of these tubulin-binding drugs, we hypothesize that agents with alternate scaffolds that bind α-tubulin could possibly overcome the drug resistance associated with β-tubulin binders. An encouraging report from Nikas et al. indicated that *TUBA3C*, a gene that encodes α-tubulin, was overexpressed in ovarian cancer patients who survived <3 years (short-term survivors) following platinum/paclitaxel chemotherapy, compared with patients who survived >7 years (long-term survivors) after treatment.[17] Thus, an α-tubulin-binding agent could significantly impact cancers that are resistant to β-tubulin-binding anticancer agents and help treat ovarian cancer patients overexpressing the *TUBA3C* gene.

The only natural product shown to bind to α-tubulin by X-ray crystallography is pironetin (**1**, Figure 1), which was isolated from *Streptomyces* strains in 1993 and 1994.[18–20] Pironetin has potent antiproliferative activity in vitro against various cancer cell lines with reported GI_{50} values of 5–8 nM.[21,22] Osada and co-workers had originally proposed that pironetin forms a covalent bond with lysine 352 of α-tubulin via conjugate addition into the α,β-unsaturated lactone.[23] However, the X-ray crystal structure of pironetin-bound α-tubulin showed a covalent adduct being formed between cysteine 316 instead of lysine 352.[24,25] Although pironetin has potent antiproliferative activity in vitro against various cancer cell lines including cell lines which overexpress P-glycoprotein[21] while maintaining inactivity against normal lung fibroblasts,[22] the natural product has not been developed as a chemotherapeutic agent.



Pironetin (**1**)

Figure 1. Structure of pironetin.

To evaluate pironetin as a potential chemotherapeutic agent, we conducted structure–activity relationship studies with a focus on the α,β-unsaturated lactone, as pironetin's mechanism of action involves a Michael addition to the lac-

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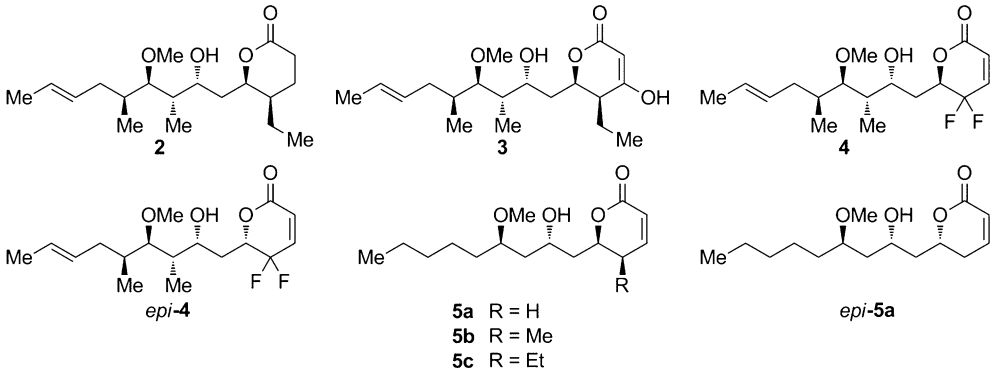


Figure 2. Structures of reported pironetin analogues with modifications at the α,β-unsaturated lactone.

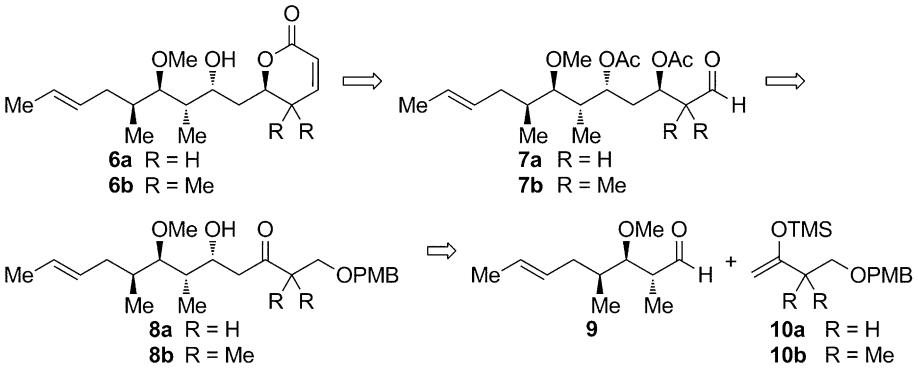
tone double bond. Previously reported pironetin analogues containing modifications at various positions of the lactone are shown in Figure 2. Kitahara and co-workers reported that analogue **2**, bearing a saturated lactone, had 1000-fold decreased activity in a microtubule disassembly assay, relative to the natural product.[26] Vogt et al. showed that the addition of a hydroxy group to the β-position of the unsaturated lactone (compound **3**) resulted in a 10–75-fold decrease in antiproliferative activity in various cancer cell lines.[22] Moreover, Qing and co-workers synthesized *gem*-difluorinated analogue **4** and the corresponding C5-epimer *epi*-**4**, and the GI_{50} values for these analogues were 600 and 1500 nM against MGC803 and A375 cancer cell lines, respectively.[27] Marco and co-workers prepared a series of simplified pironetin analogues **5** to evaluate the structure–activity relationships at the C4 and C5 positions.[21,28,29] They proposed that the C4 ethyl group is necessary for biological activity, as analogue **5c** had a GI_{50} value of 22 μM, whereas **5b** was inactive with a GI_{50} value >200 μM. The group also concluded that the stereochemistry at the C5 position did not significantly influence the biological activity of their analogues because analogue **5a** and *epi*-**5a** had GI_{50} values of 22.9 and 44 μM, respectively. While Marco and co-workers were able to explore the structure–activity relationship at the C4 and C5 positions of the lactone with their simplified scaffold, their analogues were all 1000-fold less active than pironetin in their assays.

Results and Discussion

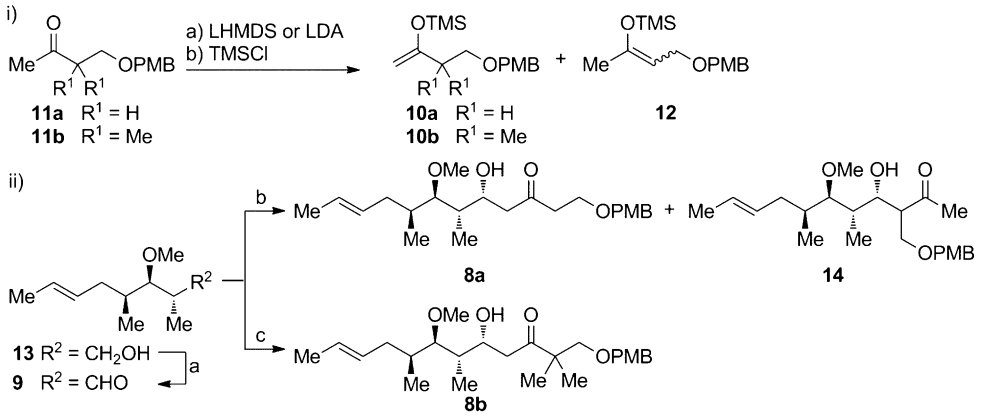
Synthesis of pironetin analogues

To explore the structure–activity relationship at the C4 and C5 positions of pironetin in more detail, we sought to synthesize and evaluate analogues that are selectively modified at the C4 and C5 positions while maintaining the remainder of the pironetin structure. We first planned the synthesis of desethyl pironetin (**6a**) and the *gem*-dimethyl analogue **6b** (Scheme 1). For the synthesis of analogues **6**, we followed Keck's pironetin total synthesis[30] starting from β-acetoxy aldehyde **7**.[31] This intermediate would be derived following functional group modification of β-hydroxy ketone **8**. Intermediate **8** would be obtained from a stereoselective Mukaiyama reaction between aldehyde **9** and silyl enol ethers **10**.

The synthesis of analogues **6** began with known alcohol **13** (Scheme 2),[30] which was oxidized to aldehyde **9** and subsequently reacted with silyl enol ethers **10** to yield β-hydroxy ketones **8**. Evans et al. developed models for the Mukaiyama aldol between silyl enol ethers and aldehydes containing either an α-substituent and/or a β-alkoxy substituent.[32] This model predicts that the addition of silyl enol ether **10** would be directed to the desired *Re* face of aldehyde **9** in the presence of $BF_3{\cdot}Et_2O$ by both the α- and β-stereocenters to yield products **8**. For the synthesis of β-hydroxy ketone **8a**, the Mu-



Scheme 1. Retrosynthesis of desethyl **6a** and *gem*-dimethyl **6b** pironetin analogues.



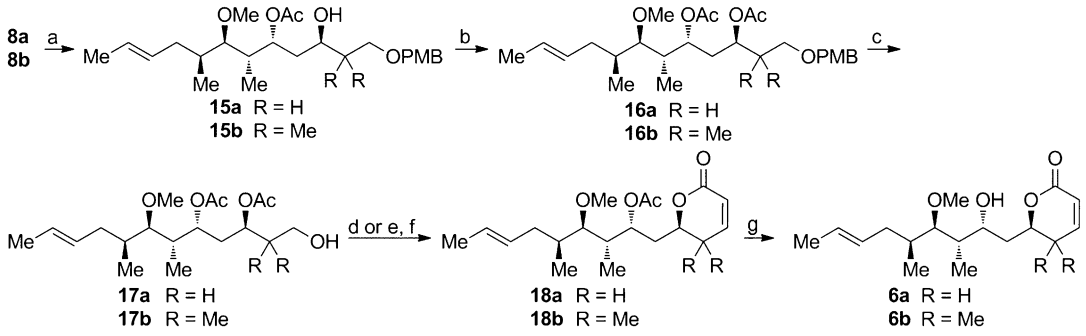
Scheme 2. Stereoselective Mukaiyama aldol reaction between aldehyde **9** and enol ethers **10** and **12**. a) cat. TPAP, NMO, CH_2Cl_2, 0 °C; b) **10a**/**12**, $BF_3{\cdot}Et_2O$, CH_2Cl_2, −90 °C, 34% over two steps for **8a** from **13**, 12% over two steps for **12** from **13**; c) **10b**, $BF_3{\cdot}Et_2O$, CH_2Cl_2, −90 °C, 52% over two steps from **13**.

kaiyama aldol was performed with a 1.1:1 mixture of silyl enol ethers **10a** and **12**, which formed as a result of kinetic and thermodynamic deprotonation of ketone **11a**. Although aldehyde **9** was treated with a mixture of silyl enol ethers **10a** and **12**, the major product was the desired product **8a**. Aldol product **14**, resulting from reaction between aldehyde **9** and silyl enol ether **12**, was isolated as a minor product.

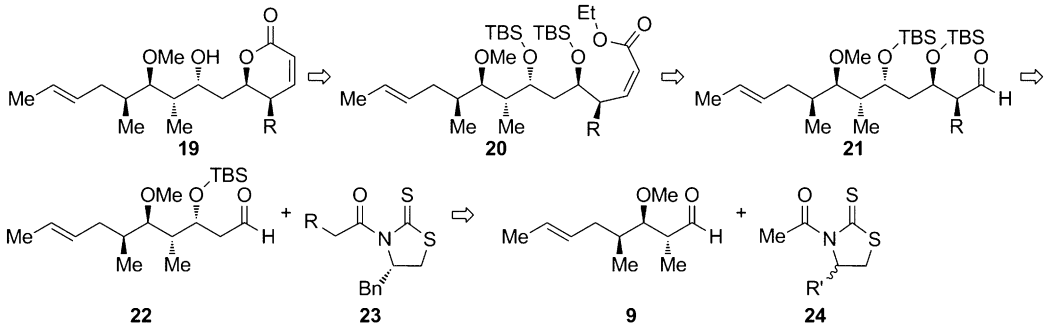
Intermediates **8** were then used to prepare the desired analogues **6** (Scheme 3).[30] A SmI_2-catalyzed *anti*-selective disproportionation between β-hydroxy ketones **8** and acetaldehyde furnished the desired intermediate **15**.[30,33] The relative configuration of intermediates **15** was assigned following hydrolysis of the acetate ester and conversion of the resulting diol into the acetonide.[34,35] Intermediates **15** were readily converted into primary alcohols **17** by protection of the secondary alcohol as the acetate and removal of the PMB protecting group. The primary alcohol was oxidized to desired aldehydes **7** and treated with the lithium enolate of methyl acetate to afford the α,β-unsaturated lactones **18**. The acetate group was hydrolyzed under acidic conditions to yield desired desethyl and *gem*-dimethyl pironetin analogues **6a** and **6b**.

To generate additional structure–activity relationship information, we synthesized pironetin analogues **19** containing a variety of C4 substituents (Scheme 4). We modified our synthetic route to more readily introduce groups at the C4 position via cyclization of intermediate **20**, which results from a *Z*-selective olefination of aldehyde **21**. Similar strategies have been used by multiple groups for the synthesis of the α,β-unsaturated lactone of pironetin.[36–41] Aldehyde **21** could be obtained from aldehyde **9** via sequential aldol reactions with the corresponding thiazolidinethiones. Crimmins and Dechert previously reported an iterative aldol/olefination/lactonization route for the total synthesis of pironetin.[40] The advantage of this synthetic route over the one used for the synthesis of analogues **6** is the ease of synthesis of thiazolidinethiones **23** to allow the introduction of different C4 groups, which in the previous synthesis would have required the preparation of the respective silyl enol ethers.

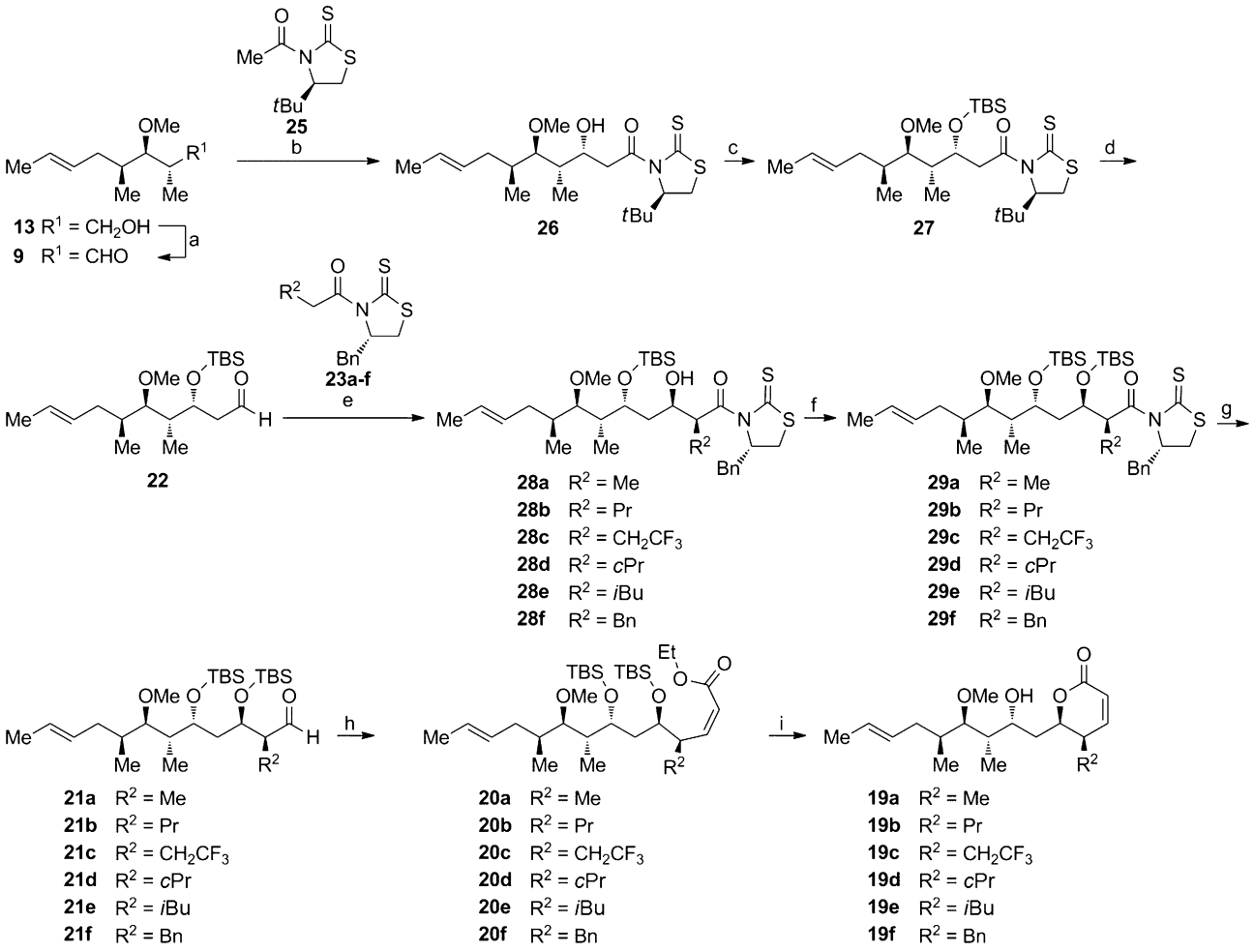
Conditions for boron and titanium enolate additions of *N*-acetyl thioazolidinethiones have been reported to occur with high diastereoselectivity.[42–44] The facial selectivity for acetate addition varies with the reaction conditions for the generation of the enolate. We chose to perform the acetate aldol with *tert*-leucine derived thiazolidinethione **25** (Scheme 5), as the thiazolidinethione precursor is readily synthesized from the commercially available unnatural amino acid.[43] The reaction between aldehyde **9** and the boron enolate of thiazolidinethione **25** proceeded in moderate yield to furnish intermediate



Scheme 3. Synthesis of analogues **6**. a) cat. SmI_2, MeCHO, THF, −20 °C, 81–96%; b) cat. DMAP, Ac_2O, TEA, CH_2Cl_2, RT, 79–99%; c) DDQ, CH_2Cl_2/H_2O, RT, 62–68%; d) cat. TPAP, NMO, CH_2Cl_2, RT; e) Dess–Martin periodinane, CH_2Cl_2, RT; f) LHMDS or LDA, methyl acetate, THF, −78 °C→RT, 24–51% over 2 steps for **18** from **17**; g) aq. HCl, MeOH, 60 °C, 23–59%.



Scheme 4. Retrosynthesis of C4-modified pironetin analogues.



Scheme 5. Synthesis of C4-modified pironetin analogues. a) cat. TPAP, NMO, CH_2Cl_2, 0 °C; b) **25**, (+)-sparteine, $PhBCl_2$, CH_2Cl_2, −78 °C, 70% over two steps from **13**; c) TBSOTf, 2,6-lutidine, CH_2Cl_2, 0 °C→RT, 89%; d) DIBAL-H, CH_2Cl_2, −78 °C, 85%; e) **23**, $TiCl_4$, DIPEA, NMP, CH_2Cl_2, −78 °C→−50 °C, 61–85%; f) TBSOTf, 2,6-lutidine, CH_2Cl_2, 0 °C→RT, 78–94%; g) DIBAL-H, CH_2Cl_2, −78 °C, 53–90%; h) ethyl di-*o*-tolylphosphonoacetate, NaH, THF, −78 °C→0 °C, 70–95%; i) aq. HCl, EtOH, RT, 43–77%.

26 (Scheme 5). Protection of the secondary alcohol as the TBS silyl ether followed by diisobutylaluminum hydride cleavage of the chiral auxiliary afforded aldehyde **22**. The various groups at the C4 position were introduced via the *syn*-aldol addition of the titanium enolate of thiazolinethione **23** to yield intermediates **28**. We primarily focused on only introducing hydrophobic groups at this position to focus our evaluation on the effect of having groups with different steric properties. Intermediates **28** were converted to aldehydes **21** following similar reaction conditions as for the conversion of intermediate **26** to aldehyde **22** for the protection of the secondary alcohol of intermediates **28** and removal of the chiral auxiliary. Under reaction conditions reported by Ando, a Horner–Wadsworth Emmons olefination between aldehyde **21** and ethyl di-*o*-tolylphospho-

noacetate afforded Z-olefin **20**.[45] We prepared a series of analogues **19a**–**19f** containing different groups at the C4 position following acid cleavage of both silyl ethers and lactonization.

Analogues containing a branched substituent at the C4 position such as cyclopropyl or isobutyl groups could be synthesized by our route; however, the synthesis of isopropyl analogue **19g** required different methodology for the synthesis of the α,β-unsaturated lactone (Scheme 6). We introduced the isopropyl group following the aldol reaction between aldehyde **22** and thiazolidinethione **23g**. The diisobutylaluminum hydride reduction of intermediate **29g**, however, resulted in only 8% of desired aldehyde **21g** along with 21% of over-reduced alcohol **30** and 62% unreacted starting material. We hypothesized that the incomplete reduction was due to the steric properties of the isopropyl group. Due to the mixture of products following diisobutylaluminum hydride cleavage, we chose to convert intermediate **29g** to alcohol **30** via lithium borohydride reduction of the thiazolidinethione amide.[46,47] The primary alcohol was subsequently oxidized to desired aldehyde **21g**. Our previous strategy for installing the α,β-unsaturated lactone via a *Z*-selective olefination and lactonization reaction was unsuitable for the isopropyl analogue. The reaction between ethyl di-*o*-tolylphosphonoacetate and aldehyde **21g** did not occur, even in the presence of ten equivalents of the phosphonate ester. The steric properties of the isopropyl group could hinder the addition of the phosphonate ester into the aldehyde; thus, we sought an alternative method for the synthesis of the α,β-unsaturated lactone involving less sterically demanding reagents. Previously, Nelson and co-workers reported the synthesis of the pironetin α,β-unsaturated lactone via a one pot ester hydrolysis, lactonization, and subsequent β-hydroxy group elimination of the corresponding β,δ ester diol.[48] The acetate aldol between aldehyde **21g** and the lithium enolate of ethyl acetate resulted in the formation of β-hydroxy ester **31**. Heating intermediate **31** in the presence of toluenesulfonic acid afforded in a one-pot silyl ether deprotection, ester hydrolysis, lactonization and elimination, the desired analogue **19g**.

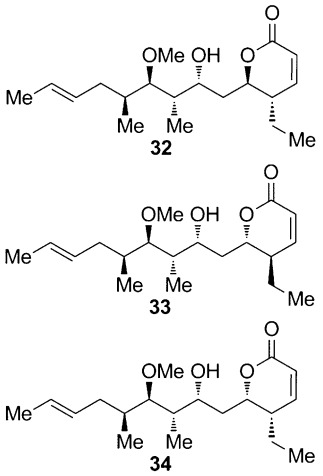
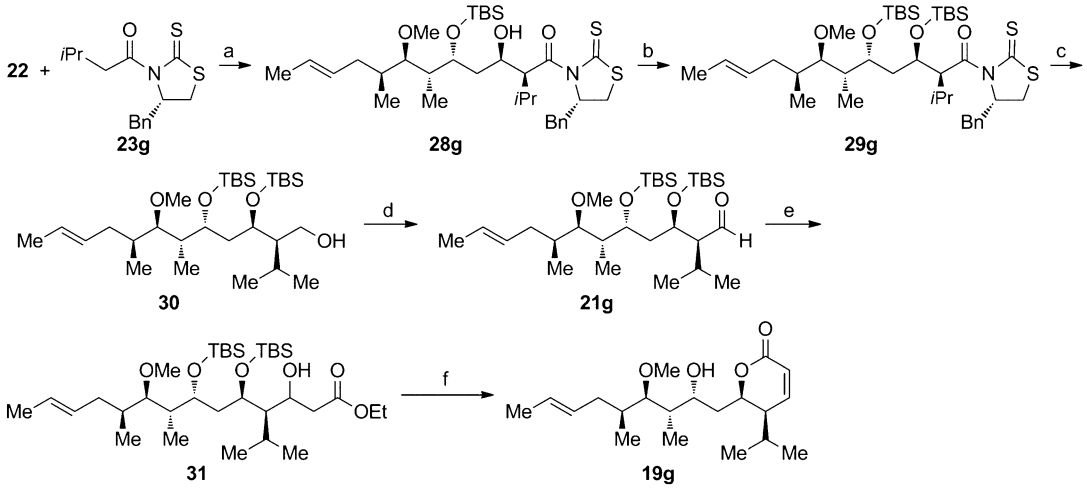
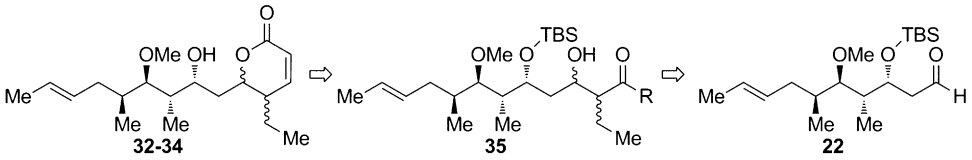


Figure 3. Structures of C4 and C5 pironetin stereoisomers.

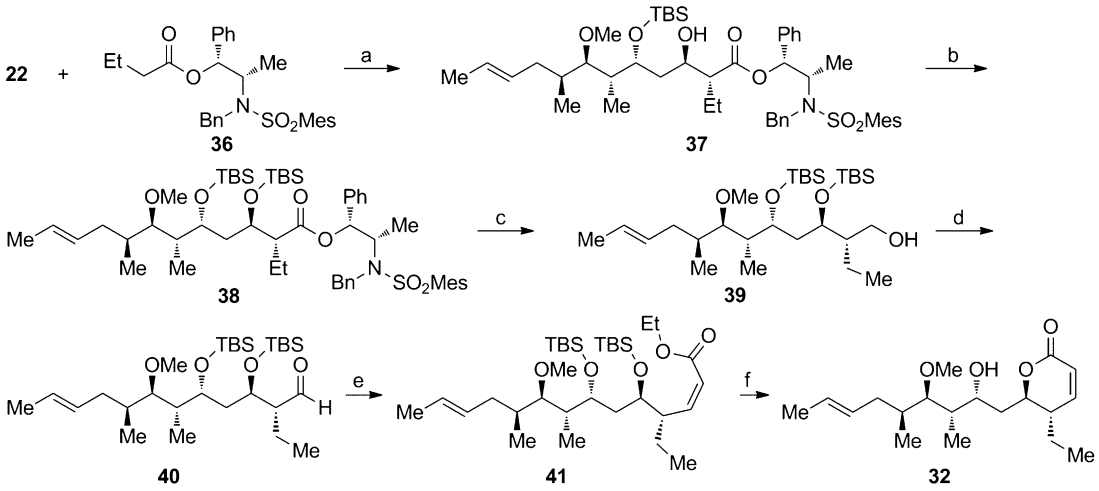
To further explore the structure–activity relationship of the α,β-unsaturated pironetin lactone, we sought to synthesize analogues **32**–**34** (Figure 3), which vary in the absolute and relative stereochemistry at the C4 and C5 positions of pironetin. The desired stereochemistry at these positions could be established via the appropriate *syn*- or *anti*-aldol reaction of aldehyde **22** as shown in Scheme 7. For the synthesis of C4-*epi*-pironetin analogue **33**, the relative stereochemistry between the C4 and C5 positions requires an *anti*-selective aldol with aldehyde **22**. While Evans and co-workers have reported the *anti*-selective aldol between thiazolidinethiones and conjugated aldehydes or benzaldehydes,[49] these conditions were not amenable for the *anti*-selective aldol with aldehyde **22**. Thus, we performed the *anti*-aldol using the norephedrine derived esters developed by Masamune and co-workers.[50] Aldehyde **22** reacted with the boron enolate of ester **36** to furnish aldol product **37** as shown in Scheme 8. Subsequent protection of the secondary alcohol as the TBS ether and diisobutylaluminum hydride reduction of the ester generated intermediate **39**. For the synthesis of the α,β-unsaturated lactone, the primary



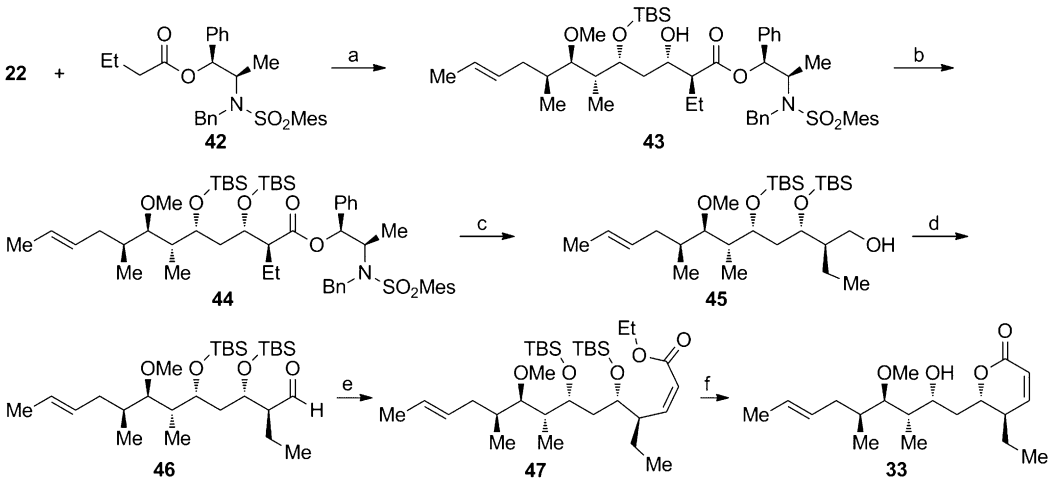
Scheme 6. Synthesis of isopropyl analogue **19g**. a) **23g**, $TiCl_4$, DIPEA, NMP, CH_2Cl_2, −78 °C→−50 °C, 76%; b) TBSOTf, 2,6-lutidine, CH_2Cl_2, 0 °C→RT; 86%; c) $LiBH_4$, MeOH, Et_2O, 0 °C, 60%; d) cat. TPAP, NMO, CH_2Cl_2, 0 °C, 74%; e) LHMDS, EtOAc, THF, −78 °C, 73%; f) TsOH, [D_8]PhMe, 110 °C, 65%.



Scheme 7. Retrosynthesis for analogues **32**–**34**.



Scheme 8. Synthesis of C4-*epi*-pironetin analogue **32**. a) Cy_2BOTf, TEA, CH_2Cl_2, −78 °C→−40 °C, 56%; b) TBSOTf, 2,6-lutidine, CH_2Cl_2, 0 °C→RT; 90%; c) DIBAL-H, CH_2Cl_2, −78 °C, 72%; d) cat. TPAP, NMO, CH_2Cl_2, 0 °C, 85%; e) ethyl di-*o*-tolylphosphonoacetate, NaH, THF, −78 °C→RT; 57%; f) aq. HCl, EtOH, RT, 44%.



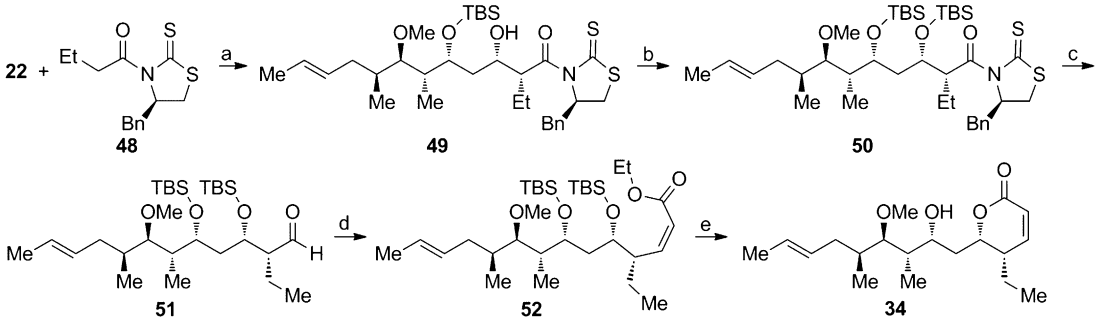
Scheme 9. Synthesis of C5-*epi*-pironetin analogue **33**. a) Cy_2BOTf, TEA, CH_2Cl_2, −78 °C→−40 °C, 66%; b) TBSOTf, 2,6-lutidine, CH_2Cl_2, 0 °C→RT; 85%; c) DIBAL-H, CH_2Cl_2, −78 °C, 88%; d) cat. TPAP, NMO, CH_2Cl_2, 0 °C, 86%; e) ethyl di-*o*-tolylphosphonoacetate, NaH, THF, −78 °C→RT; 74%; f) aq. HCl, EtOH, RT, 25%.

alcohol was oxidized to aldehyde **40** and carried forward to analogue **32** following *Z*-selective olefination and subsequent lactonization. As shown in Scheme 9, employing aldehyde **22** and ester **42**, the C5-*epi*-pironetin analogue **33** was synthesized following the same route.

Because the C4,C5-*epi*-pironetin analogue **34** contains a *syn*-relationship between the C4 and C5 positions, thiazolidinethione based *syn*-aldol methodology could be applicable for the synthesis of the desired analogue. Aldol reaction between aldehyde **22** and thiazolidinethione **48** established the desired stereochemistry at these positions, as shown in Scheme 10. Intermediate **49** was carried on to desired analogue **34** following the previous synthetic route involving lactone synthesis via a *Z*-selective olefination followed by lactonization.

Antiproliferative activity of pironetin analogues

To evaluate the activity of the new analogues, we tested each compound for antiproliferative activity against the OVCAR5 ovarian cancer cell line. The calculated GI_{50} values for each ana-



Scheme 10. Synthesis of C4- and C5-*epi*-pironetin analogue **34**. a) $TiCl_4$, DIPEA, NMP, CH_2Cl_2, −78 °C→−50 °C, 75%; b) TBSOTf, 2,6-lutidine, CH_2Cl_2, 0 °C→RT, 76%; c) DIBAL-H, CH_2Cl_2, −78 °C, 51%; d) ethyl di-*o*-tolylphosphonoacetate, NaH, THF, −78 °C→0 °C, 72%; e) aq. HCl, EtOH, RT, 13%.

logue after a 48 h incubation with OVCAR5 cells are listed in Table 1.

Pironetin (entry 2) showed antiproliferative activity similar to paclitaxel (entry 1) with 22 and 17 nM GI_{50} values, respectively. The desethyl analogue **6a** (entry 3), *gem*-dimethyl analogue **6b** (entry 4) and the C4-*epi* analogue **32** (entry 12) were significantly less active than the parent compound and suggests a requirement for a single substituent at the C4 position with the same absolute stereochemistry as the natural product. Some substitution is tolerated at the C4 position, with small groups such as the methyl group (entry 5) or larger groups such as the isobutyl group (entry 9). The benzyl group (entry 10) resulted in greatly decreased activity, whereas analogues with cyclopropyl (entry 8) and propyl (entry 6) groups showed only slightly reduced activity. Isopropyl analogue **19g** (entry 11), however, had a 100-fold decrease in activity compared to pironetin. Modifying the stereochemistry at the C5 position resulted in loss of activity as shown by the high GI_{50} values for C5-*epi* pironetin **33** (entry 13) and C4,C5-*epi*-pironetin **34** (entry 14). Unlike previous studies by Marco et al. with simplified analogues **5**,[21] we found that modification of the C5 position stereochemistry is not tolerated. Our results are consistent with the X-ray structure of pironetin bound to tubulin, which shows that the C4 ethyl group of pironetin binds to a narrow hydrophobic pocket in the binding site that is unlikely to accommodate large C4 substituents, disubstituted C4 analogues, or changes in the C4 and C5 stereochemistry. Therefore, we investigated whether molecular modeling could be used as a tool for the design of future analogues. Analogues **6**, **19** and **32**–**34** were docked into the pironetin binding site in α-tubulin.[24,25] Because pironetin is a covalent inhibitor, docking scores were calculated using the CovDock module in the Schrödinger Maestro software package.[51] While we were able to dock our analogues into the binding site, a correlation was unfortunately not observed between the CovDock scores and the observed antiproliferative activity.[34]

Conclusions

We synthesized a series of pironetin analogues with modifications at the C4 and C5 positions of pironetin and evaluated their antiproliferative activity. Analogues containing either

Table 1. Antiproliferative activity of pironetin and related analogues against OVCAR5 ovarian cancer cells.

Entry	Compound		R^1	R^2	GI_{50} [nM][a]
1		paclitaxel	–	–	16.6 ± 2.1
2		pironetin (**1**)	Et	H	21.9 ± 2.5
3		**6a**	H	H	> 10000
4		**6b**	Me	Me	> 100000
5		**19a**	Me	H	182 ± 24
6		**19b**	*n*Pr	H	67.9 ± 4.0
7		**19c**	CH_2CF_3	H	371 ± 53
8		**19d**	*c*Pr	H	56.2 ± 1.6
9		**19e**	*i*Bu	H	128 ± 12
10		**19f**	Bn	H	> 10000
11		**19g**	*i*Pr	H	2050 ± 326
12		**32**	H	Et	> 33000
13		**33**	Et	H	> 30000
14		**34**	H	Et	> 30000

[a] Values are the average ± SEM of two experiments performed in triplicate ($n = 6$).

a propyl or cyclopropyl group at the C4 position showed antiproliferative activity against the OVCAR5 ovarian cancer cell line at nanomolar concentrations, but larger moieties such as isopropyl, benzyl, or trifluoroethyl cannot be tolerated at this position. We also found that modifying the stereochemistry at the C4 and C5 positions causes loss of activity. These results suggest that the configuration of the α,β-unsaturated lactone is also important for biological activity.

Experimental Section

See the Supporting Information, which contains experimental procedures, protocols, compound characterization data, and NMR spectra of all new compounds. It also contains the procedure for acetonide synthesis from intermediate **15** and corresponding NMR spectra; HPLC methods and analyses for compounds **6**, **19**, and **32**–**34**; and covalent docking protocols and results for compounds **6**, **19**, **32**–**34**.

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Conflict of interest

The authors declare no conflict of interest.

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