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Restoration of Microtubule Interaction and Cytotoxicity in D-*seco* Taxanes upon Incorporation of 20-Hydroxymethyl-4-allyloxy Groups

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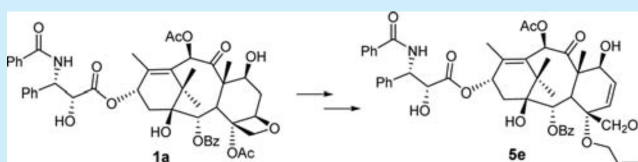
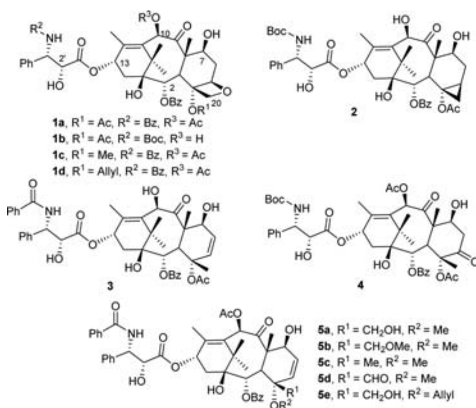
S *Supporting Information*

ABSTRACT: To probe the exact role of the oxetane D ring in both tubulin binding and cytotoxicity of taxanes, novel D-*seco* taxanes bearing a C4 ether substituent have been prepared from paclitaxel **1a**. Among them, 20-hydroxymethyl-4-allyloxy D-*seco* taxane **5e** is the most active in both tubulin and cytotoxicity assays. It is only slightly less potent than **1a** on tubulin polymerization promotion *in vitro* and the most cytotoxic among all D-*seco* taxanes known to date. The reason for the loss and restoration of bioactivity for these D-*seco* taxanes is also discussed with the assistance of NMR and molecular modeling studies. From these results, we draw a conclusion that the intact D ring of taxanes is not strictly necessary for their binding to tubulin and cytotoxic effects.



Taxane antitumor drugs, such as paclitaxel (Taxol) **1a** and docetaxel **1b**, have been widely used in clinics for more than two decades. Although numerous medicinal chemistry studies have been reported, there are still some questions to be answered regarding the ligand−protein interaction for this group of molecules. A better understanding of the interaction of taxanes with their targets, i.e. β-tubulin in microtubule (MT), in atomic detail, may give rise to the structure-based design of a new generation of taxanes with improved activity.



One remaining unanswered question is the exact role of the oxetane D ring. This unique four-membered ring was initially believed to be essential for the bioactivity of **1a**. However, more recent studies cast doubts on this belief. For example, 5(20)-deoxydocetaxel **2**[1] and the 4-methyl D-*seco* analog **3**[2] are almost as potent as their parent compounds in the tubulin/MT assays. Based on the above-mentioned studies, it was proposed that the D ring is not critical for tubulin binding if the overall conformation of the core structure and the location of the C4 substituent relative to that at C13 are preserved. When the structure of the complex between **1a** and Zn^{2+}-stabilized tubulin sheets was solved at 3.5 Å resolution,[3] the oxygen atom in the D ring was shown to establish a H-bond with Thr276 of β-tubulin. Nonetheless, its removal (as in **2** and **3**) was not followed by a dramatic decrease in the power to promote tubulin polymerization or to inhibit MT disassembly. In contrast, a very significant drop in cytotoxicity (2−3 orders of magnitude) was observed for most D-*seco* taxanes. Even the most cytotoxic one, compound **2**, is still 100-fold weaker than its counterpart **1b**.[4] Although it was suggested that this discrepancy may originate from cell membrane permeability and stability, no evidence was available to prove these suspicions. Therefore, it is reasonable to propose that the C5 oxygen atom may play a critical role for taxanes exerting cytotoxicity. In fact, the D-*seco* derivative **4**, on which an oxygen atom was reinstalled at the C5 position, was synthesized to prove this hypothesis. However, though it is only 3.5-fold weaker than **1a** for the MT disassembly inhibition, it is unable to rescue the lost cytotoxicity (1000-fold weaker than **1b**).[4]

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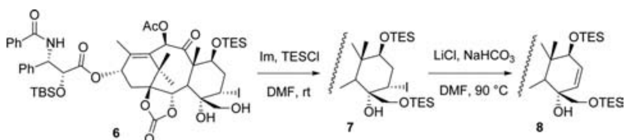
To determine the real contribution of the D ring to the bioactivities of taxanes, some C4 and C20 functionalized D-*seco* analogs **5a–e** were designed and synthesized. At the very beginning, reinstatement of an acetoxy group to C4 was attempted, but was found unfeasible due to undesired acyl migration between the C4 and C20 hydroxyls. As the published structure–activity relationships (SAR) on C4 in taxanes revealed that the cytotoxicity of 4-methoxy-4-deacetyl paclitaxel **1c** was only slightly weaker than that of **1a**,[5] the D-*seco* taxanes **5a–d** bearing 4-OMe were prepared as surrogates at the beginning.

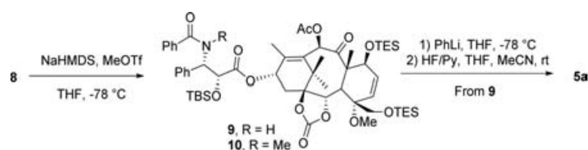
Initially, we attempted to introduce the 4-OMe prior to the oxetane ring opening. However, this changed the conformation of the C ring (data not shown), thereby preventing the elimination of the 5-I. Hence, based on the literature protocol,[6] intermediate **6** was synthesized from **1a** for the follow-up studies. After the protection of the 20-OH with a triethylsilyl (TES) group, the elimination of the iodo atom in compound **7** was realized by using the LiCl/$NaHCO_3$ system in DMF, to give the desired product in high yield (91%) (Scheme 1) according to the similar synthetic strategy developed in our lab.[7] The selection of the protective group at C20 was important, since the elimination could not occur if 20-OH was masked with other groups, such as *p*-toluenesulfonyl or methylthiomethyl.

Scheme 1. Synthesis of Compound 8



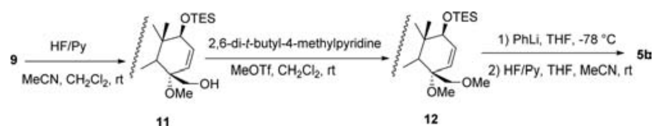
The methylation of 4-OH on **8** seemed more difficult than that in the preparation of **1c**. After many trials, the most optimal conditions were found using the NaHMDS and MeOTf system in THF (Scheme 2) to afford **9** in 88% yield (based on 65% conversion). The C3′ amide was also methylated yielding **10** if the reaction conditions were not carefully controlled. After the opening of the 1,2-carbonate with PhLi and subsequent desilylation, D-*seco* taxane **5a** was smoothly obtained in 71% yield after two steps. In contrast, it was found that the removal of the 2′-*O*-TBS in **10** was unexpectedly difficult due to the steric effect introduced by the 3′-N methyl (data not shown).

Scheme 2. Synthesis of Compound 5a



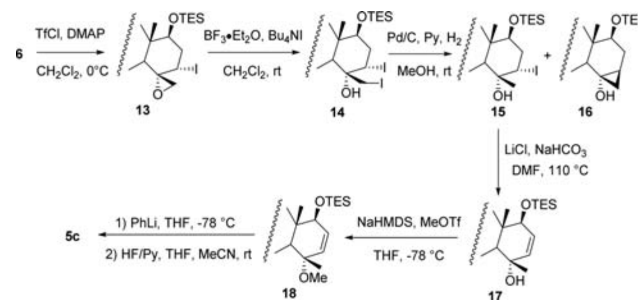
Compound **5b** was prepared from **9**, as illustrated in Scheme 3. After selective deprotection of the 20-*O*-TES group, **11** was treated with 2,6-di-*tert*-butyl-4-methylpyridine and MeOTf to afford **12**, which was converted to the desired product **5b** over two steps in moderate yield (35%).

Scheme 3. Synthesis of Compound 5b



The C20 methyl analog **5c** was synthesized from **6** (Scheme 4) by a modified procedure of that reported.[2] In accordance with the literature, catalytic hydrogenation of di-iodo D-*seco* taxane **14** using PtO_2 as the catalyst gave no reaction.

Scheme 4. Synthesis of Compound 5c



Hydrogenation over Pd/C gave both the desired product **15** and the cyclopropyl analog **16**. Referring to the literature,[8] the formation of **16** might be through a palladium-catalyzed intramolecular cyclodehalogenation. Synthesis of the known compound **17** from **15** also failed using the reported conditions,[2] but could be realized using the previously mentioned LiCl/$NaHCO_3$ conditions. Compound **5c** was prepared by a procedure similar to that used for the preparation of **5a**. In addition, the known compound **1c** was also prepared as a control for the bioassays according to the literature procedure.[4]

However, targeted compound **5d** was not obtained through either the oxidation of the 20-OH of **5a** or other synthetic strategies because the 4-formyl compound is not stable even when stored below 0 °C. From our experience in the preparation of 4-formyl analogs, the aldehyde group is prone to the attack of the C2 free hydroxyl group, either upon formation by hydrolysis of the attached acyl group or after migration of the acyl group from C2 to C1.

As shown in Table 1, the tubulin binding affinity and cytotoxicity toward A2780 cells of all 4-OMe D-*seco* compounds **5a–c** were found to be much lower (12–61-fold and 2–3 orders of magnitude, respectively) than those of **1c**. Of note, these decreases are significantly higher than those brought about by replacement of the 4-OAc in **1a** with the 4-OMe in **1c** (4- and 33-fold, respectively, relative to **1a**).

Table 1. Tubulin Binding Affinity and Cytotoxicity of the D-*seco* Analogs

compound	tubulin binding (K_b 35 °C, 10^7 M^{-1})	cytotoxicity (IC_{50}, nM) A2780
1a	1.43 ± 0.17	2.4
1c	0.38 ± 0.07	80
1d	2.20 ± 0.06	2.5
5a	0.031 ± 0.007	14 885
5b	0.0062 ± 0.0065	25 180
5c	0.0082 ± 0.0015	4350
5e	0.38 ± 0.02	42.9

To understand the poor activity of **5a–c**, we first focused on their NMR parameters, which reflect the possible conformational changes in the core structure and thus the orientation of C13 and C4 side chains. It must be borne in mind that opening

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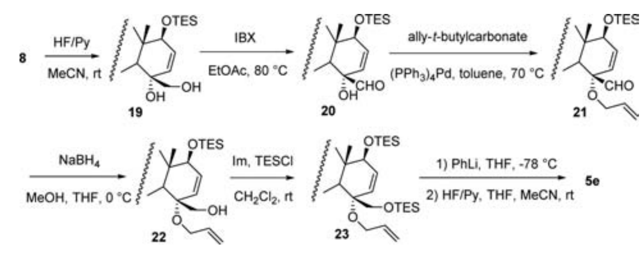
of the D ring brings about a change of puckering in the C ring (Figure S1). The major differences between **1c** and **5a−c**, as revealed by the NMR data (Table S1), are the chemical shifts of protons 14α and 14β, which appear >0.2 ppm upfield for the former and >0.1 ppm downfield for the latter, as well as the $J_{13,14\alpha}$ values (difference >1 Hz). The latter indicate that the H13−C13−C14−H14α dihedral angle related to the puckering of the A ring is significantly different in **5a−c** from that in **1a**. Indeed, in the quantum mechanically optimized structures, this angle in the **5a−c** series is consistently smaller than that in **1a** and this slightly affects the relative orientation of the C13 side chain. Support for these refined geometries is provided by the good agreement between the $J_{13,14\alpha}$ values computed using the Karplus equation and the experimental measurements (Table S2).

In addition, the divergence between tubulin binding affinity and cytotoxicity, as that of **2** and **3**, still remained for D-*seco* taxanes **5a−c**. While **5a** was about 46 times less active than **1a** in the tubulin binding assay, it exhibited more than 6200 times less cytotoxicity against A2780 cells. This suggests that the intact oxetane ring is important for the cytotoxicity of taxanes as previously thought. In light of this, we then asked whether we could further potentiate both the binding affinity and the cytotoxicity through modification of the C4 substituent.

The results of C4 extended aliphatic esters and carbonates possessing comparable or superior activity in tubulin polymerization and cytotoxicity assays[9] demonstrated that extension of the C4 substituent may lead to improved tubulin binding and cell killing activities. For this reason, the 4-allyloxy D-*seco* taxane **5e** was designed for revising the conformation of the entire molecule and strengthening the hydrophobic interaction of the C4 substituent within the binding pocket.

Following a similar approach to synthesize a C,D-spiro taxane in our lab,[7] the allylation of 4-OH could only be realized in the presence of $(PPh_3)_4Pd$ and ally-*tert*-butylcarbonate, since it was found difficult to occur by using similar conditions as methylation (Scheme 5). However, from compound **8**, the

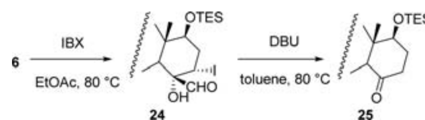
Scheme 5. Synthesis of Compound 5e



reaction occurred exclusively at the 3′-N amide (data not shown). We also attempted the reaction from the C4 allyl carbonate to avoid a side reaction, but the *in situ* conversion rate from the carbonate to the allyl ether was not satisfactory. Finally, alkylation of 4-OH only succeeded after the steric hindrance at the C20 position was reduced by oxidation to an aldehyde **20** with NMO/TPAP (yield 65%) or 2-iodoxybenzoic acid (IBX) (yield 91%). Then, the C4 allyloxy analog **21** was obtained in 95% yield without formation of the C3′-N allyl byproduct. After the C20 aldehyde group in **21** was reduced by $NaBH_4$, the freshly generated hydroxyl was concealed by a TES group. Eventually, the desired product **5e** was obtained in 70% yield over last two steps. In addition, the C4-allyloxy ether analog **1d** with the intact oxetane D ring was also prepared as a reference compound for the bioassays (Scheme S1).

We also tried to oxidize the 20-OH of **6** before the elimination of the 5-I to shorten the synthetic steps. However, after **24** was treated under various elimination conditions, only an unexpected product **25** was obtained near-quantitatively (Scheme 6). This observation suggested that an intramolecular

Scheme 6. Synthesis of Unexpected Compound 25



reaction precedes the intermolecular reaction in the presence of a catalytic base or acid. In addition, after the C4 hydroxyl of **24** had been substituted, the eliminated product could be obtained by $LiCl/NaHCO_3$ methods, but accompanied by a C5 chlorinated byproduct (1:1) (data not shown). This indicated that reducing the hindrance at C20 could compromise the partial untoward effect from the C4 substitution. From these results, the plausible mechanisms for the occurrence of **25** are proposed (Scheme S2).

The D-*seco* taxane **5e** exhibited a binding affinity similar to that of **1c** and was even 2-fold more cytotoxic against A2780 cells than **1c** (Table 1). Therefore, it was much more potent than its 4-methoxy counterpart **5a** in both tubulin binding (12-fold) and cytotoxicity assay (350-fold). It is also noteworthy that the resistant/sensitive (*R*/*S*) ratio of cytotoxicity for **5e** is lower than that of **1a** on both A2780AD/A2780 and HeLa/βIII/HeLa cell lines, although the absolute potency is still much weaker than that of **1a** (Table S3).

Contrary to the previous thought that the intact oxetane D ring is crucial for cytotoxicity,[4] we found that 20-hydroxylation combined with attachment of a hydrophobic chain of proper length (e.g., allyloxy) to C4 not only enhances the binding affinity and promotes tubulin polymerization (only 2.4-fold weaker than **1a**) but also increases the cytotoxicity to a great extent (only 18-fold less cytotoxic than **1a**).

The NMR data for **5e**, when compared to the data for **1c**, showed the same tendency that was observed for **5a−c** in the 4-OMe D-*seco* series but to a lesser extent (Table S1). In fact, the value of the H13−C13−C14−H14α dihedral angle for **5e** (141.4°, Table S2) was intermediate between those measured for **1a** (147.5°) and **5a−c** (∼135°). However, **5e** behaved as **1c** in molecular and cellular assays rather than as **5a−c** did.

This contradiction could be interpreted by a molecular modeling study. Inspection of the most up-to-date molecular model of the complex of **1a** with tubulin[10] reveals a very tight fit between the bulky drug and the taxane-binding site (Figure 1). At the bottom of this large pocket the oxygen of the oxetane ring acts as a H-bond acceptor for the backbone NH of Thr276. The 4-OMe D-*seco* **5a−c** derivatives, which differ from **1a** and **1b** in the puckering of ring C, cannot establish this interaction. But when the R^1 substituent at C20 is a hydroxymethyl (as in **5a**), this oxygen can presumably act as a H-bond donor for the backbone CO of Thr276 instead. This interaction is reinforced when an allyloxy group is present at C4 (as in **5e**) because this extended group, relative to a methoxy (as in **1c**) or an acetoxy (as in **1a**), improves the van der Waals contacts with the phenyl ring of Phe272. If it is absent, however, the strength of the H-bond involving the 20-hydroxymethyl is not enough to

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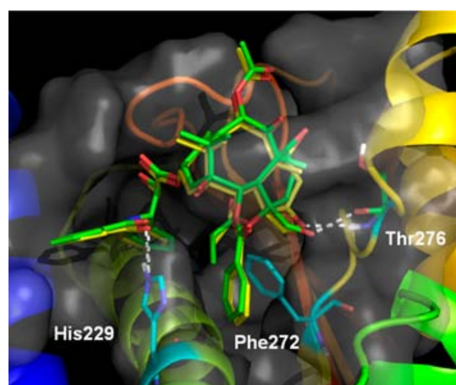

Figure 1. Taxane-binding site in β-tubulin showing bound **1a** (C atoms in yellow) and **5e** (C atoms in green).

compensate for the worse fit and the lack of interaction with NH of Thr276. Furthermore, if the substituent at C20 lacks the OH, the binding affinity drops dramatically because neither a methyl group (as in **5c**) nor a methoxy group (as in **5b**) can be accommodated in the polar and relatively small subpocket that lodges the oxetane of **1a** in β-tubulin. The beneficial effect of the allyloxy group over an acetoxy on C4 is also manifested in taxane **1d**, which displays greater affinity for tubulin and is slightly more cytotoxic on HeLa cells than **1a** (Table 1 and Table S3).

In general, both opening of the oxetane D ring and shortening of 4-OAc to 4-OMe decreased the interaction with tubulin and also the cytotoxicity. The biological activity was almost abolished in 4-OH and -H analogs.[11] The substituents attached to C4, on the other hand, extend deep into the cavity and are directed toward the floor of the tubulin binding pocket, resulting in either no contact with the floor or, if long enough, a hydrophobic interaction that improves binding provided there no steric clash. There is an exception to the lack of C4 substituent contact as described above, namely the encounter between the C4 unit and water molecules that occupy the binding site in the absence of ligand.

Apart from the above-mentioned enthalpy considerations, the enhanced bioactivity of **5e** can also be explained through an entropy-driven process. The taxane-tubulin binding pocket is an expansive cavity capable of binding a wide variety of antitubulin drugs[12] as well as a pool of water molecules in the absence of ligand. MT binding of such molecules can lead to either favorable or unfavorable entropy contributions to ΔG, as demonstrated by Buey et al.[13] It can be hypothesized that the improvement of the binding and cytotoxic action of compound **5e** relative to the other D-*seco* compounds in Table 1 results from penetration of the allyloxy group into the tubulin binding pocket leading to the displacement of one or more tightly bound water molecules. The accompanying favorable ligand desolvation entropy can be enhanced by installation of C4 moieties that are able to drive out additional water molecules and, thereby, improve the tubulin binding affinity and cytotoxic potential of the D-*seco* class of agents.

In conclusion, the present results demonstrate that an intact D ring in taxanes is not strictly necessary for their interaction with tubulin and for exerting their cytotoxic effects.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b03119.

Complete experimental procedures, product characterization, spectroscopic data for all new compounds, and other experimental details (PDF)

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Notes

The authors declare no competing financial interest.

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