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Potent Taccalonolides, AF and AJ, Inform Significant Structure−Activity Relationships and Tubulin as the Binding Site of These Microtubule Stabilizers

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Supporting Information

ABSTRACT: The taccalonolides are a class of microtubule stabilizing agents isolated from plants of the genus *Tacca*. In efforts to define their structure−activity relationships, we isolated five new taccalonolides, AC−AF and H2, from one fraction of an ethanol extract of *Tacca plantaginea.* The structures were elucidated using a combination of spectroscopic methods, including 1D and 2D NMR and HR-ESI-MS. Taccalonolide AJ, an epoxidation product of taccalonolide B, was generated by semisynthesis. Five of these taccalonolides demonstrated cellular microtubule-stabilizing activities and antiproliferative actions against cancer cells, with taccalonolide AJ exhibiting the highest potency with an IC_{50} value of 4.2 nM. The range of potencies of these compounds, from 4.2 nM to >50 μM, for the first time provides the opportunity to identify specific structural moieties crucial for potent biological activities as well as those that impede optimal cellular effects. In mechanistic assays, taccalonolides AF and AJ stimulated the polymerization of purified tubulin, an activity that had not previously been observed for taccalonolides A and B, providing the first evidence that this class of microtubule stabilizers can interact directly with tubulin/microtubules. Taccalonolides AF and AJ were able to enhance tubulin polymerization to the same extent as paclitaxel but exhibited a distinct kinetic profile, suggesting a distinct binding mode or the possibility of a new binding site. The potencies of taccalonolides AF and AJ and their direct interaction with tubulin, together with the previous excellent in vivo antitumor activity of this class, reveal the potential of the taccalonolides as new anticancer agents.

For over 40 years, drugs that target microtubules have been important in oncology. In 2010, two of the four new drugs approved by the FDA for the treatment of cancer targeted microtubules, suggesting that microtubules remain a valuable target for anticancer drugs. Paclitaxel, the first microtubule stabilizer identified, was isolated from *Taxus brevifolia*. Although it has achieved significant clinical success, the limitations of paclitaxel and the second-generation analogue docetaxel include intrinsic and acquired multidrug resistance and dose-limiting toxicities, prompting the development of new classes of microtubule-stabilizing drugs.[1,2] Recently, two microtubule stabilizers that bind within the taxane site, cabazitaxel and the epothilone ixabepilone, have been approved for clinical use. These drugs can circumvent some but not all of the limitations of the first- and second-generation microtubule stabilizers.[3,4]

In our search for microtubule-disrupting agents, we identified a new class of microtubule stabilizers, the taccalonolides, from the tropical plant *Tacca chantrieri*.[5] The taccalonolides have highly acetylated pentacyclic steroidal skeletons and are structurally distinct from other microtubule stabilizers. The cellular effects of the most abundant taccalonolide, A (**1**), are almost identical to the effects of paclitaxel. They both increase the density of cellular microtubules, cause the formation of aberrant mitotic spindles leading to mitotic arrest and apoptosis, and have excellent antitumor efficacy in vivo.[5,6] However, multiple experimental approaches were unsuccessful in identifying a direct interaction between **1** and tubulin/microtubules.[7,8] Other studies showed that the taccalonolides A, E, B (**2**), and N can circumvent clinically relevant forms of taxane resistance, including expression of P-glycoprotein and βIII tubulin, suggesting that the taccalonolides offer advantages over existing microtubule-targeting agents.[6] In a recent study, we isolated three additional taccalonolides with antimitotic and microtubule-stabilizing properties (designated as Z, AA, and AB) and began to identify structure−activity relationships (SARs) for this class of compounds.[9] This communication focuses on the isolation and characterization of five additional naturally occurring taccalonolides, designated as AC (**3**), AD (**4**), AE (**5**), AF (**6**), and H2 (**8**), and one semisynthetic product, AJ (**7**), which allow significant refinement of the SARs for this class of molecules. Additionally, the single-digit nanomolar potency of **7** gave us the ability to detect a direct interaction between a taccalonolide and tubulin.

The structures of the taccalonolides (Figure 1) were solved using 1D and 2D NMR spectra as well as high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) data. All of the compounds were obtained as white powders. The ^{1}H spectra of **3**−**6** and **8** revealed signals for five methyls, four acetyl-group methyls, four oxygenated methines, two epoxide methines, and one olefinic methine (Supplemental Table 1), which are characteristic of the taccalonolides. The molecular formula of compound **3** was determined to be $C_{36}H_{46}O_{16}$ by HR-ESI-MS

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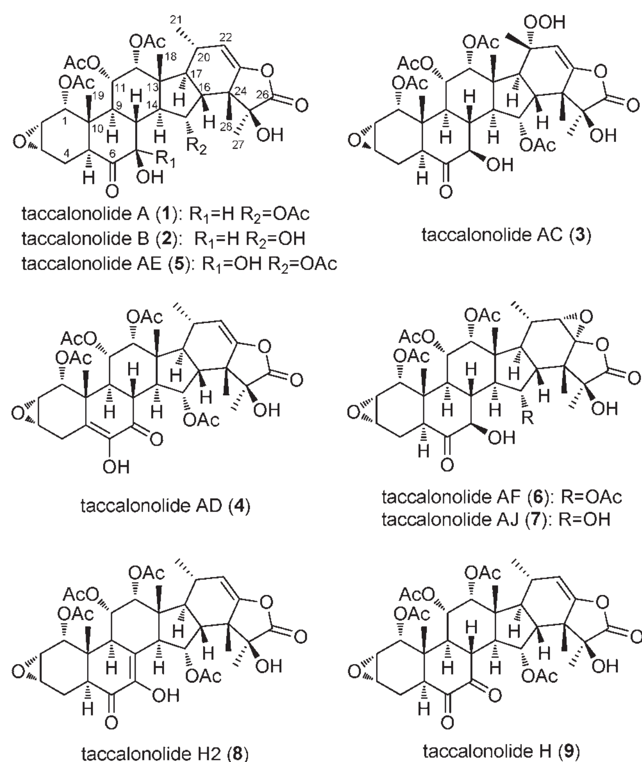

Figure 1. Taccalonolide structures.

analysis (calcd m/z 735.2864, exptl 735.2893), which contains two more O atoms than taccalonolide A (**1**). The proton NMR spectrum of **3** showed a singlet resonance for the C21 methyl group, indicating a quaternary C20, which requires an additional substituent at C20 in comparison with **1**. This was confirmed by the heteronuclear multiple-bond correlations (HMBCs) between H22 and C17/C20/C24 and between H21 and C17/C20/C22. The substitution was proposed to be a hydroperoxyl group on the basis of the chemical shift of C20 at 84.5 ppm and the two additional oxygen atoms required by the molecular formula. Additional support for the hydroperoxyl group was provided by the downfield shift of C20 relative to that of taccalonolide W, which possesses a hydroxyl group at C20.[10] All of the taccalonolides discovered to date possess 18β-Me and 16β-H configurations. The α-orientation of the hydroperoxyl group was deduced by the nuclear Overhauser effect (NOE) correlations between Me21 and H16/Me18. The other signals of **3** are similar to those of **1**, so the structure of **3** depicted in Figure 1 was proposed and the trivial name taccalonolide AC given.

Liquid chromatography (LC)–ESI-MS of **4** showed pseudomolecular ions at m/z 701 $[M + H]^+$, 718 $[M + NH_4]^+$, and 723 $[M + Na]^+$, indicating a mass 2 Da less than that of **1**. The molecular formula of **4** was determined to be $C_{36}H_{44}O_{14}$ by HR-ESI-MS analysis (calcd m/z 701.2807, exptl 701.2787). The ^{13}C NMR spectrum showed two additional olefinic carbon signals at 143.9 and 127.3 ppm, suggesting the presence of one more double bond in **4** than in **1**. The location of this double bond between C5 and C6 was deduced by the HMBCs between C5 and H1/H3/H19. In addition, an enol hydroxyl group at the C6 position was determined by the HMBCs between the hydroxyl proton at 6.26 ppm and C6/C7. The shift of the ketone group to C7 was evidenced by the HMBC between C7 at 190.3 ppm and H8/H9/6-OH. Therefore, the structure of compound **4** depicted in Figure 1 was determined and the trivial name taccalonolide AD assigned.

Compound **5** gave pseudomolecular ions at m/z 719 $[M + H]^+$, 736 $[M + NH_4]^+$, and 741 $[M + Na]^+$ in LC–ESI-MS. HR-ESI-MS of **5** showed an $[M + H]^+$ ion at m/z 719.2944 (calcd 719.2915), corresponding to a molecular formula of $C_{36}H_{46}O_{15}$, which differs from that of **1** by one additional O atom. The ^1H and ^{13}C NMR spectra of **5** were very similar to those of **1**. The only difference was the occurrence of a signal for an additional hydroxyl group at 5.01 ppm instead of H7 at ca. 4 ppm. The location of the additional hydroxyl group at C7 to form a geminal diol was deduced by the chemical shift of C7 at 92.4 ppm and confirmed by the HMBCs between 7-OH at 5.01 ppm and C6/C7/C8, between 7-OH at 3.64 ppm and C6/C7, and between H8 and C7. Compound **5** represents a rare example of geminal diol functionality for natural products and is designated as taccalonolide AE. It was stable during our experiments.

The molecular formula $C_{36}H_{46}O_{15}$ was established for compound **6** by the $[M + H]^+$ ion at m/z 719.2911 (calcd 719.2915) in the HR-ESI-MS spectrum, indicating the presence of one more O than is found in **1**. The ^1H and ^{13}C NMR data for **6**, which were fully assigned through 2D NMR experiments, closely resembled those of **1**. The only difference was the absence of resonances for the C22–C23 olefin and the appearance of signals for one oxygenated methine (δ_H = 3.29 ppm and δ_C = 65.9 ppm) and one quaternary carbon at 92.2 ppm. In accordance with the molecular formula of **6**, the additional oxygen should be present as an epoxide group at C22/C23. This was confirmed by the HMBCs between H21 and C20/C22, between H22 and C20/C21, and between H28 and C23/C24. The relative configuration of this epoxide group was deduced from the coupling constants. The small $^3J_{H20/H22}$ coupling constant requires an equatorial (β-oriented) H22, so the epoxide group is α-oriented. This epoxide-containing taccalonolide was given the trivial name taccalonolide AF.

Compound **8** showed the same pseudomolecular ions as **4**, at m/z 701 $[M + H]^+$, 718 $[M + NH_4]^+$, and 723 $[M + Na]^+$. The molecular formula was determined to be $C_{36}H_{44}O_{14}$ by HR-ESI-MS analysis (calcd m/z 701.2809, exptl 701.2813). Similar to **4**, compound **8** also showed carbon signals for an additional double bond at 140 and 126 ppm in its ^{13}C NMR spectrum. The location of this additional double bond was determined to be C7/C8 on the basis of the HMBCs between H9/H14 and C7 and between H9/H14/H15 and C8. A hydroxyl group resonance at 6.27 ppm showed HMBCs with C6/C7/C8, suggesting that this hydroxyl group is located at C6. Thus, the structure of **8** was determined to be the enol of the previously reported taccalonolide H (**9**).[11] Since **8** is stable as the enol, the trivial name taccalonolide H2 was given to distinguish it from taccalonolide H containing the ketone.

Compounds **1**–**5** and **8** were isolated in sufficient quantities to allow accurate determination of their biological activities. Taccalonolide AF (**6**) was present at low abundance in the extract, and more material was semisynthesized by epoxidation of the abundant compound **1** using dimethyldioxirane.[12] The reaction was carried out quantitatively under very mild conditions and gave **6** as the single product. No β-epoxide isomer was observed. After evaporation of the solvent and reagent, quantities of **6** sufficient for biological studies were obtained. Because of the potent activity of **6**, taccalonolide AJ (**7**), the epoxidation product of taccalonolide B (**2**), was also produced using the same method.

Table 1. Antiproliferative Potencies of the Taccalonolides

compound	IC_{50} (nM)[a]
taccalonolide A (**1**)	5380 ± 230
taccalonolide B (**2**)	3120 ± 180
taccalonolide AC (**3**)	>50000
taccalonolide AD (**4**)	3480 ± 230
taccalonolide AE (**5**)	5010 ± 210
taccalonolide AF (**6**)	23 ± 3
taccalonolide AJ (**7**)	4.2 ± 0.3
taccalonolide H2 (**8**)	730 ± 20
paclitaxel	1.0 ± 0.1

[a] Concentrations of taccalonolides that caused 50% inhibition of cellular proliferation, as measured in HeLa cells using the SRB assay (n = 3).

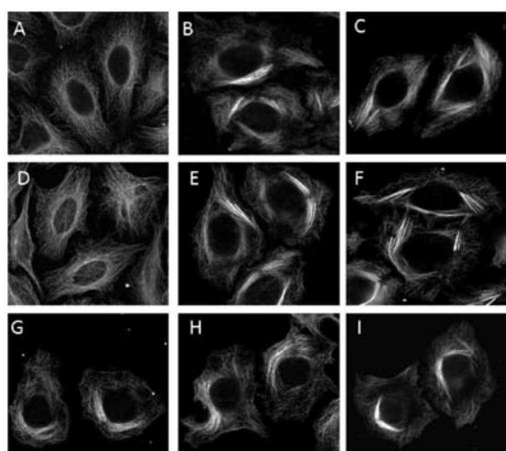

Figure 2. Effects of the taccalonolides on interphase microtubules. HeLa cells were treated with vehicle or a taccalonolide for 18 h. Shown are representative images of cells treated with (A) vehicle, (B) **1**, (C) **2**, (D) **3**, (E) **4**, (F) **5**, (G) **6**, (H) **7**, and (I) **8**. Concentrations equal to 5 times the IC_{50} were used for all of the taccalonolides except **3**, which was added at 20 μM. Microtubules were visualized by indirect immunofluorescence using a β-tubulin antibody.

Except for **3**, all of the taccalonolides that were isolated or synthesized inhibited the proliferation of and exhibited cytotoxicity toward HeLa cells, with IC_{50} values ranging from 4.2 nM for **7** to 5 μM for **1** and **5** (Table 1). In contrast, concentrations of **3** up to 50 μM had no effect on cellular proliferation. Significant increases in cellular microtubule density and microtubule bundling occurred at concentrations 5-fold greater than the IC_{50} for each of the active compounds, consistent with the microtubule-stabilizing activities of other taccalonolides (Figure 2). In contrast, concentrations of **3** up to 20 μM showed no effects on cellular microtubules (Figure 2). In addition to increasing the density of interphase microtubules, all of these taccalonolides except **3** caused HeLa cells to arrest in the G_2/M phase of the cell cycle (Supplemental Figure 2) with multiple aberrant mitotic spindles (Supplemental Figure 3), which are additional phenotypes associated with microtubule-stabilizing agents. No change in cell cycle distribution was observed in cells treated with a 20 μM concentration of **3** (Supplemental Figure 2). In immunofluorescence studies, **3**-treated cells in mitosis exhibited normal bipolar spindles (Supplemental Figure 3) and DNA alignment (data not shown). These data are consistent with the inability of **3** to disrupt interphase microtubules and inhibit cell proliferation. Compound **3** appeared to be relatively stable under the assay conditions, reducing the possibility that its inactivity was due to rapid decomposition.

The large number of naturally occurring taccalonolides distinguishes them as one of only a few natural product classes where the SARs can be identified without significant synthetic manipulation. Our previous studies with other taccalonolides provided initial indications of SARs.[6,9] A bulky isovalerate at the C1 position, found in taccalonolide T, was associated with excellent potency relative to **1**.[9] Additionally, upon comparison of **1**, **2**, and taccalonolides E and N, the presence or absence of acetoxy groups at C11 or C15 did not have a major effect on potency.[6] When comparing the potency of other taccalonolides, however, the presence or absence of the C11 acetoxy group and the hydrolysis of the C15 acetate group were contingent on moieties present in other parts of the molecule, suggesting interrelations across the northern and southern regions.[9] In the present study, **8** showed a 7.4-fold increase in potency relative to **1**. The only difference between these two taccalonolides is the presence of an additional double bond in **8** at C7/C8. The location of this double bond is important, since a double bond at C5/C6 (as in **4**) does not provide the same increase in potency. When a hydroxyl group was added to C7 of **1** to form the rare geminal diol in **5**, the potency was also unchanged (Table 1). Surprisingly, **3**, which differs from **1** only by an additional hydroperoxyl group at C20, showed no antiproliferative activity at concentrations up to 50 μM, suggesting the importance of substituents at this site. In contrast, **6** and **7**, which differ from **1** and **2** by conversion of the C22−C23 double bond to an epoxide group, increased the activity 234−743-fold, giving the most potent taccalonolides identified to date (Table 1). These results clearly suggested that an epoxide moiety at C22/C23 provides optimal potency, and we will test this further with additional taccalonolides in future studies. Taken together, these results highlight the importance of the C20,C22−C23 region of the taccalonolide molecule and suggest that this region plays a central role in its interactions with tubulin.

Previously, all classes of microtubule-stabilizing agents that increase the density of cellular microtubules except the taccalonolides were shown to stimulate the polymerization of purified tubulin in biochemical preparations, demonstrating a direct interaction with tubulin.[7] Multiple studies evaluating the interactions of **1** and **2** with purified tubulin, microtubule protein, or cellular lysates containing tubulin and microtubule-associated proteins suggested that these taccalonolides are not able to bind directly to tubulin.[7,8] However, these studies were all performed with **1** and **2**, which have antiproliferative potencies in the low micromolar range. In this study we generated **7**, the first taccalonolide to inhibit cellular proliferation at concentrations less than 5 nM, the range of potency of microtubule stabilizers in clinical use. In contrast to all previous studies with **1** and **2**, we found that **7** enhanced both the rate and extent of purified tubulin polymerization (Figure 3). A direct comparison of the effects of **7** and paclitaxel on tubulin polymerization at equimolar concentrations showed that the two drugs are able to polymerize tubulin to the same extent. However, a lag in the time required for polymerization to occur was observed in the presence of **7**, as opposed to the immediate tubulin assembly that occurs with paclitaxel. This suggests that **7** may interact with tubulin/microtubules in a manner distinct from that for paclitaxel or bind to a different binding site. Studies with the other highly potent taccalonolide, **6**, indicated that it is also able to enhance the

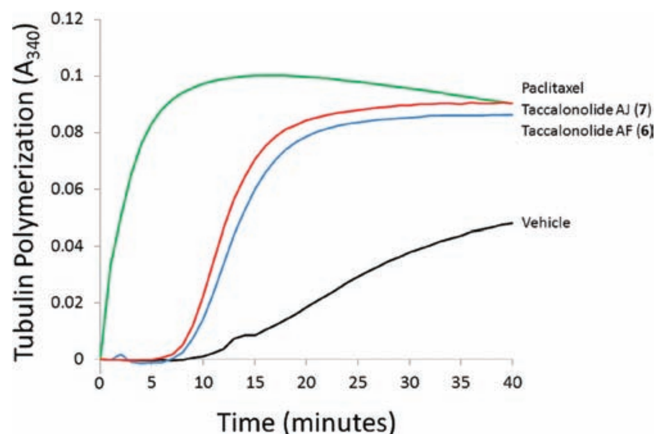

Figure 3. Effects of **6** and **7** on tubulin assembly. Purified porcine brain tubulin was incubated at 37 °C in the presence of vehicle or 10 μM paclitaxel, **6**, or **7**. Microtubule polymerization was monitored turbidimetrically at 340 nm.

polymerization of purified tubulin (Figure 3), demonstrating that this is a common feature for taccalonolides with low-nanomolar potency. Additional experiments to test this hypothesis and define the interaction between the taccalonolides and tubulin are ongoing and will provide important information regarding the mechanism of action of this class of microtubule stabilizers and whether they bind within the taxane site, the peloruside A/laulimalide site, or a unique site on microtubules.

Taccalonolides **4** and **8** are ketone/enol tautomers of **9**. Additionally, **5** and **9** are potentially interconvertible geminal diol and ketone moieties (Supplemental Figure 4). It is interesting that we isolated all of them as stable isomers, as suggested by the different biological potencies obtained with these compounds. Additionally, significant tautomerization or interconversions were not seen during purification or storage in the NMR solvent over 3 weeks. The enol and germinal diol in **4**, **5**, and **8** may be stabilized by the α-carbonyl and 15-acetoxy groups through intramolecular H-bonds. Since quantities were limited, the conditions for tautomerization and interconversion were not evaluated but will be investigated in future studies.

The six new taccalonolides described in this study have expanded the SARs of this class and identified the critical importance of the E-ring constituents at C20−C23. The C22/C23 epoxide group in **6** and **7** dramatically increases the potency, while the C20 hydroperoxyl group in **3** results in total loss of antiproliferative and microtubule-stabilizing activities.

Most notable is the finding that taccalonolides with single-digit nanomolar potency can be identified and that compounds **6** and **7**, the most potent taccalonolides isolated to date, afforded us the ability to detect a direct interaction between these taccalonolides and purified tubulin, consistent with their actions in cells. The rationale for why **6** and **7** interact with purified tubulin whereas **1** and **2** cannot affect tubulin polymerization is likely related to differences in potency. The ability of microtubule-targeted agents to affect the polymerization of purified tubulin in biochemical assays requires concentrations that are orders of magnitude higher than the concentration required to observe changes in microtubule density and antiproliferative and antimitotic effects in cells. A number of factors contribute to this discrepancy, including the ability of these compounds to be concentrated hundreds of fold across the cell membrane.[13,14] Additionally, in intact cells, microtubule-associated proteins modulate the effects of these agents. The current findings demonstrate that the potent taccalonolides **6** and **7** interact directly with tubulin. Future studies to identify the binding site and nature of the interactions of the taccalonolides with tubulin and/or microtubules will be performed. It is interesting to speculate on the possibility of a third microtubule stabilizer binding site. The potent biological activities of **6** and **7**, their interaction with tubulin, and the excellent in vivo antitumor effects of other taccalonolides, together with the proven value of microtubule-stabilizing drugs, clearly reveal the potential of the taccalonolides as anticancer agents.

■ ASSOCIATED CONTENT

Supporting Information. Experimental details, 1D and 2D NMR spectra, and additional bioassay data. This material is available free of charge via the Internet at http://pubs.acs.org.

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