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Design, synthesis and biological evaluation of a simplified fluorescently labeled discodermolide as a molecular probe to study the binding of discodermolide to tubulin

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ABSTRACT

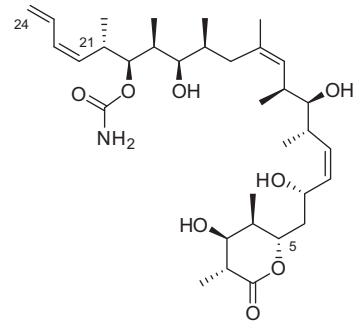
The design, synthesis, and biological evaluation of a simplified fluorescently labeled discodermolide analogue possessing a dimethylaminobenzoyl fluorophore has been achieved. Stereoselective Suzuki coupling and Horner–Wadsworth–Emmons reaction comprised the key tactics for its construction. The analogue exhibited qualitatively similar activity to paclitaxel in a tubulin assembly assay, and it can thus be used as a fluorescent molecular probe to explore the local environment of the discodermolide binding site on tubulin. The results of fluorescence measurements on the tubulin-bound analogue are reported.

1. Introduction

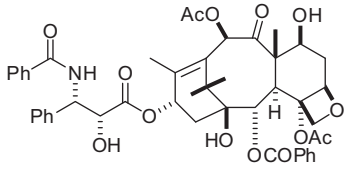
The polyketide natural product discodermolide (**1**, Fig. 1) was isolated from the marine sponge *Discodermia dissoluta*.[1] It had potent cytotoxicity to human and murine cell lines,[1] and its mechanism of action was found to be very similar to that of the important anticancer drug paclitaxel (**2**, Fig. 1). It promotes the assembly and stabilization of microtubules, causing cell cycle arrest at the G2/M phase boundary and subsequent cell death by apoptosis.[2]

These facts made discodermolide a prime candidate for preclinical and eventually clinical development. The low yield of discodermolide from its producing organism, and the difficulty of aquaculture approaches, made synthesis the only viable route to the quantities needed for this work. Several effective synthetic routes have been developed, including practical large-scale syntheses by the Smith[3] and Paterson groups[4]; these studies have been reviewed by Smith and Freeze.[5] Extensive preclinical studies were carried out by Novartis, using material prepared by modifications of these routes,[6] and initial Phase I clinical results were encouraging.[7] Sadly further development was discontinued because of lack of efficacy and toxicity issues,[8] but it is possible that modified analogues might still prove useful anticancer drugs.

If further development of discodermolide is to become a reality, it would be most useful to have a detailed understanding of the



discodermolide (**1**)



paclitaxel (**2**)

Figure 1. Discodermolide (**1**) and paclitaxel (**2**).

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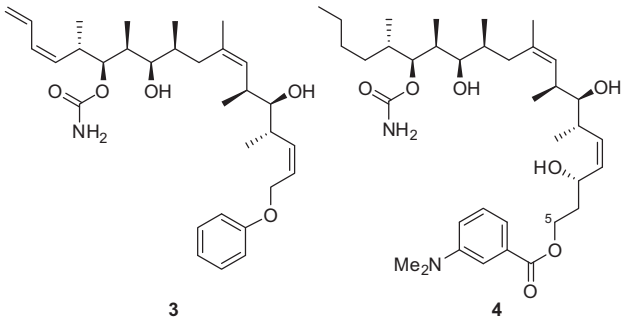


Figure 2. Simplified discodermolides **3** and **4**.

binding of discodermolide to the tubulin polymer, and in particular to know the binding conformation of discodermolide. Unlike paclitaxel and other tubulin-binding agents with polycyclic or macrocyclic skeletons, discodermolide consists of a flexible chain, and thus can adopt a seemingly infinite number of conformations both in solution and when binding to tubulin. Since the discodermolide-tubulin complex has not been crystallized, the nature of the binding conformation must be determined by indirect methods. Extensive NMR studies on the conformation of free discodermolide or tubulin-bound discodermolide have been conducted, and have provided valuable but not fully conclusive information on the question.[9] A recent study by Horwitz and co-workers probed hydrogen–deuterium exchange between tubulin and discodermolide by mass spectrometry. This study indicated that unlike paclitaxel, which interacts primarily with the M-loop of tubulin, discodermolide orients itself towards the N-terminal H1-S2 loop.[10] Finally, the combination of force-field conformational searches with NMR deconvolution in different solvents led to the conclusion that the hairpin conformation of discodermolide predominates. Docking studies then identified two distinct docking poses, and one of them was identified as better accommodating the available SAR data.[11]

In addition to these NMR and mass spectrometric methods, indirect evidence of the orientation of a ligand in the tubulin binding pocket can be obtained from the fluorescence energy transfer spectroscopy of the ligand-protein complex.[12] Fluorescence spectroscopy is widely used to study ligand–receptor interactions and the local environment of a binding site.[13] At this time such studies have not been reported on discodermolide, although the synthesis of a fluorescent discodermolide derivative has been reported by Smith.[14] In this paper, we describe the synthesis and biological evaluation of two simplified fluorescently labeled discodermolide analogues as potential fluorescent molecular probes to explore the local environment of the discodermolide-tubulin binding site by fluorescence spectroscopy.

In previous studies in our group, the small environmentally sensitive dimethylaminobenzoyl fluorophore was successfully used to explore the local environment of the paclitaxel binding site on microtubules.[15] In the case of paclitaxel, this group could replace the side chain *N*-benzoyl group or the C-2 *O*-benzoyl group with minimal impact on the overall structure and activity, but the incorporation of such a fluorophore into discodermolide must inevitably cause major structural changes, which could lead to the loss of activity. We thus explored incorporating the fluorophore as a replacement for the δ-lactone ring of discodermolide, and were encouraged by the fact that several analogues of this nature, including the simple analogue **3**, had submicromolar cytotoxic activity against various cell lines (Fig. 2).[5] Analogue **4** was thus designed to accomplish this (Fig. 2).

2. Results and discussion

2.1. Synthesis of fluorescent discodermolide analogue 4

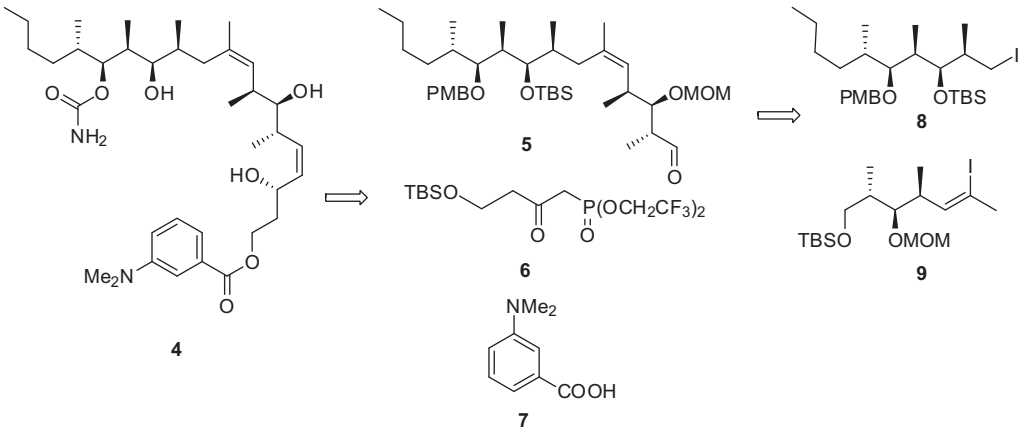
In the design of analogue **4** the terminal diene at the C21-24 position was replaced by a saturated butyl group to simplify the synthesis, since this conjugated diene system does not contribute significantly to the activity of discodermolide.[14,16] The retrosynthetic analysis of analogue **4** is shown in Scheme 1.

The synthesis of analogue **4** involved the final installation of the fluorophore **7** preceded by the Horner–Wadsworth–Emmons coupling of the phosphonate **6** and aldehyde **5**, which was prepared from iodide **8** and vinyl iodide **9**by Suzuki coupling.

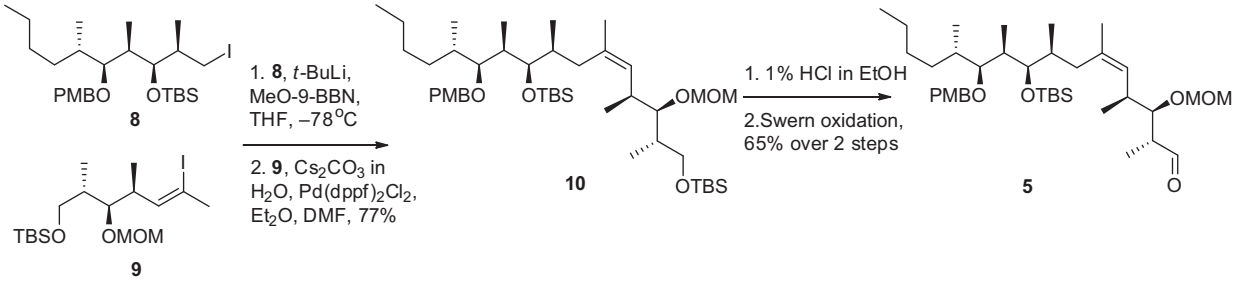
The precursors **8** and **9** were synthesized by the convergent approach developed by Smith and his group.[16b,17] Their connection was achieved via Suzuki coupling to afford TBS ether **10** in 77% yield. Subsequent TBS deprotection and Swern oxidation provided aldehyde **5** in 65% yield (Scheme 2).

The synthesis of subunit **6** began with propane-1,3-diol, which was mono protected as its TBS ether, and then oxidized by Dess–Martin oxidation and further oxidation by $NaClO_2$ and NaH_2PO_4 to give acid **11**. This was then converted to acid chloride **12** by treatment with 1-chloro-*N*,*N*,2-trimethyl-1-propenylamine,[18] and **12** was transformed to phosphonate **6** in 40% yield over two steps (Scheme 3).

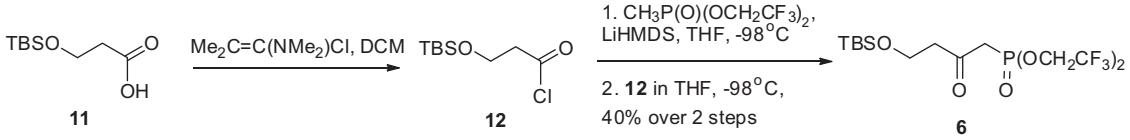
As shown in Scheme 4, HWE coupling between subunits **5** and **6** afforded TBS ether **13** in 85% yield, which was then subjected to



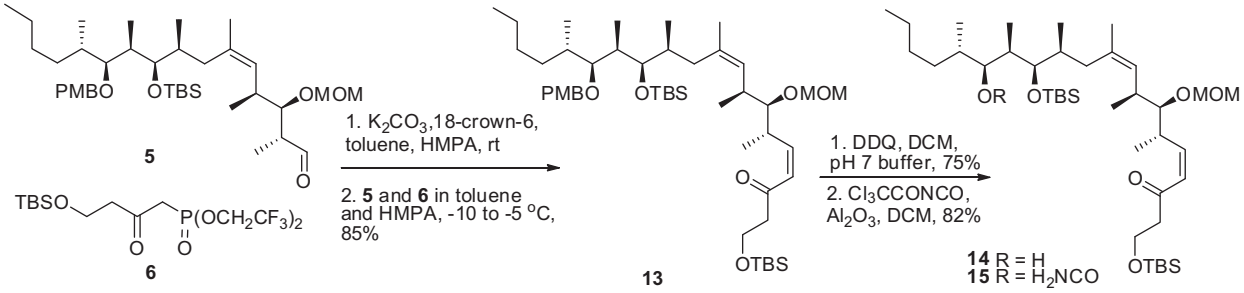
Scheme 1. Retrosynthesis of analogue **4**.



Scheme 2. Synthesis of aldehyde **5**.



Scheme 3. Synthesis of phosphonate **6**.



Scheme 4. Synthesis of fragment **15**.

oxidation with DDQ to remove the PMB protecting group to give alcohol **14**. Subsequent installation of the carbamate moiety on alcohol **14** gave carbamate**15** (Scheme 4).

Reduction of the keto group of carbamate **15** by K-selectride™ gave alcohol **16**, whose configuration was determined by Mosher ester analysis.[19] Alcohol **16** was coupled separately with *R*-2-methoxyphenylacetic acid (*R*-MPA) and *S*-MPA to form the *S* and *R* Mosher esters via EDC coupling (Scheme 5). Their ^{1}H NMR spectra were obtained and the δ^{R-S} values were calculated for adjacent protons to the chiral center. The results are summarized in Figure 3, where the δ^{R-S} values of the top part of the structure were positive, while the value for the bottom part was negative. According to the Mosher ester model (Fig. 3), the configuration was confirmed to be the desired *S*.

Compound **16** was then treated with HCl (4 N) in small portions over 4 h to remove all the protecting groups, and the resulting alcohol **17** was coupled with 3-dimethylaminobenzoic acid via EDC coupling to give the final product **4** in 16% yield over two steps (Scheme 6).

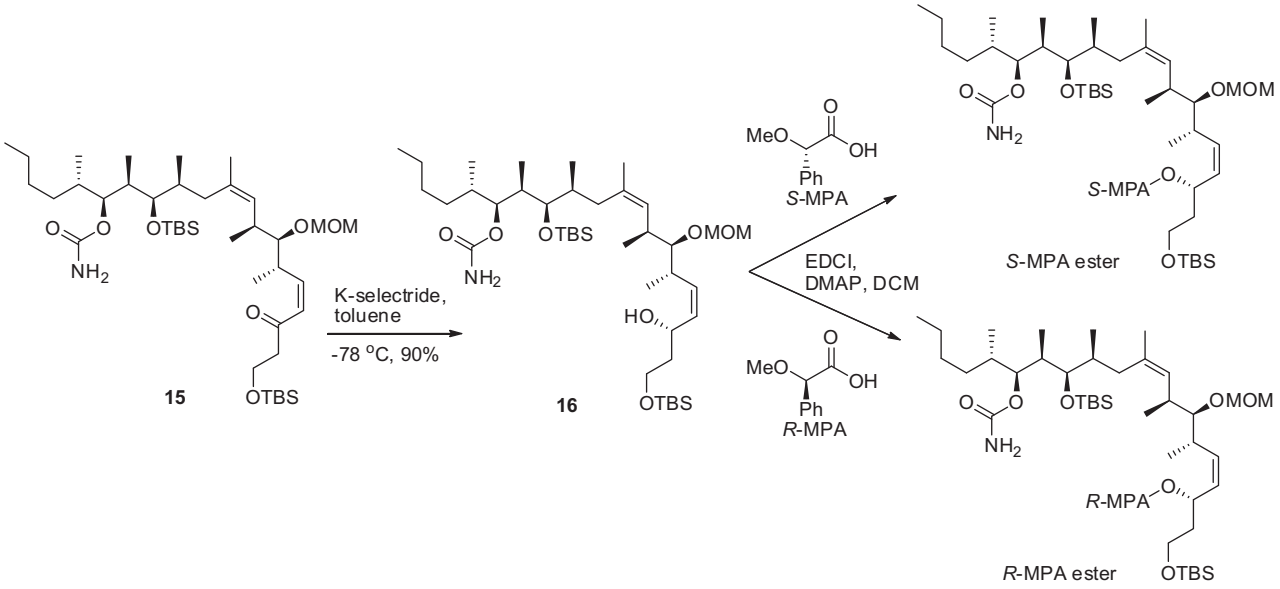
2.2. Biological evaluation of fluorescent discodermolide analogue

The simplified, fluorescently-labeled discodermolide analogue **4** was evaluated for antiproliferative, tubulin assembly and tubulin binding activities. The assay results are summarized in Table 1. Analogue **4** is significantly less active than paclitaxel in the A2780 and PC3 assays, respectively. Compound **4** is almost 400-fold less toxic than paclitaxel toward A2780 cells. This relative activity in the cells is reflected in the tubulin assembly activity, since compound **4** is an order of magnitude less potent than paclitaxel in promoting microtubule assembly.

The emission energy and intensity of the *m*-dimethylamino benzoate fluorophore is sensitive to solvent polarity.[21] This fluorophore has been used previously to probe the paclitaxel site on tubulin by Sengupta et al.,[22] who also noted that the emission maximum of this probe in various solvents was affected by both the polarity of the solvent and its hydrogen bonding capabilities. We evaluated the emission maximum and intensity of **4** in various solvents (Table 2). In general, decreasing the polarity of the solvent increased the emission intensity and shifted the emission maximum to shorter wavelength. We were unable to observe fluorescence of the probe in buffer. This type of behavior has been noted in *m*-aminobenzonitriles, whose fluorescence properties are very similar to those of *m*-amino benzoates.[21,23] We observed very weak fluorescence of the molecules when 2% DMSO was included in the buffer. Increasing the concentration of DMSO in the buffer increased the emission intensity to detectable levels.

As hoped, tubulin binding induced a large increase in the emission intensity of the environmentally sensitive dimethylamino benzoate fluorophore for **4** (Fig. 4). The increase in emission intensity and the shorter wavelength of the emission maximum point to a hydrophobic environment for the microtubule-bound fluorophore.

The fluorescent signal was used to determine the affinity of the compound for GMPcPP-stabilized microtubules. Plots of the increase in ligand fluorescence as a function of concentration displayed saturation behavior and were fit a single rectangular hyperbola. The dissociation constant for **4** binding to microtubules



Scheme 5. Reduction of fragment **15** and Mosher ester formation from alcohol **16**.

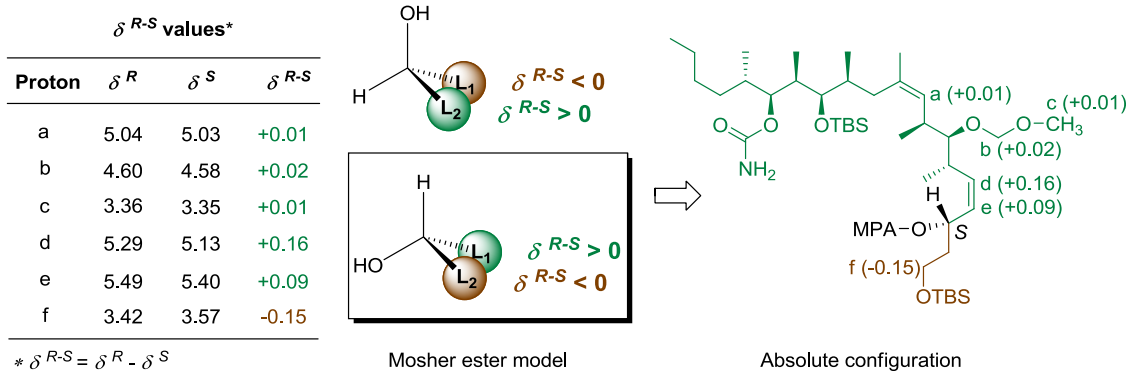
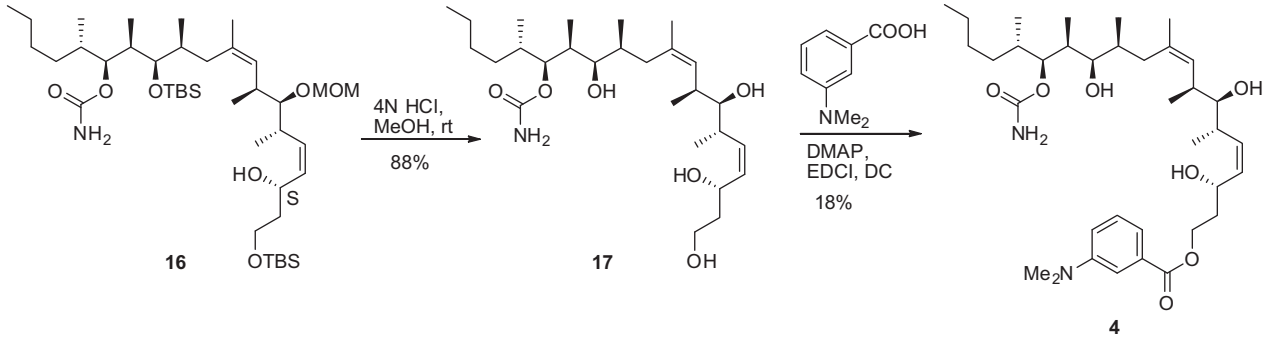


Figure 3. Determination of the absolute configuration of **16**.



Scheme 6. Finale of the synthesis of analogue **4**.

Table 1
Cytotoxicity and tubulin binding activity of analogue **4**

Compound	A2780 assay IC_{50} (nM)	Tubulin assembly assay EC_{50} (μM)	Binding assay K_d (μM)
Paclitaxel **2**	14 ± 2	0.49 ± 0.22	0.015 ± 0.004[a]
4	5400 ± 220	5.2 ± 1.2	24 ± 5

[a] From Li et al.[20]

is 1600-fold greater than that of paclitaxel (Table 1). Since the lactone moiety of discodermolide is contained within the protein binding site, it is not surprising that a significant structural modification at this position decreases the affinity of a ligand for this

Table 2
Emission maxima of **4** in different environments

Solvent[a]	λ_{max}[a] (nm)	RFI[b]
Dioxane	431	15
DMSO	439	12
MeCN	443	12
EtOH	453	3
MeOH	460	1
4 with microtubules	438	—[c]

[a] Excitation at 345 nm.
[b] Relative fluorescence intensity (RFI) at emission maximum.
[c] RFI not meaningful in this context.

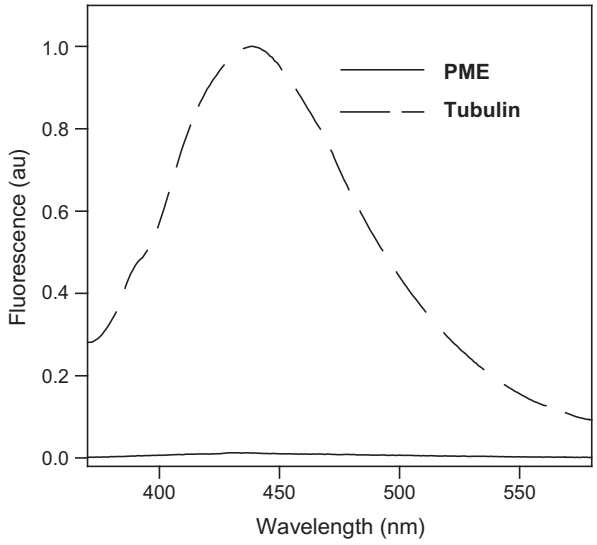


Figure 4. Representative fluorescence spectra of analogue **4** in the presence and absence of tubulin. The spectra shown are of 2 μM **4** in PME with 4% v/v DMSO alone (solid line) and with 10 μM tubulin (dashed line). The excitation wavelength was 345 nm. Background fluorescence due to buffer and protein were subtracted.

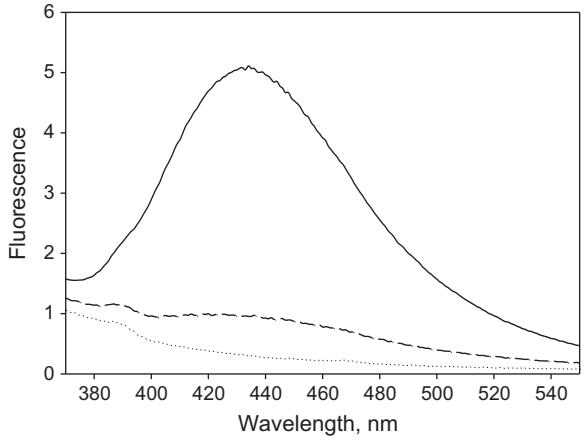


Figure 5. Fluorescence of **4** bound to microtubules in the presence and absence of paclitaxel. Tubulin (10 μM) in PME containing 1 mM GTP was incubated with 90 μM **4** at 37 °C. Paclitaxel was added to a total concentration of 10 μM. The final concentration of DMSO was 4% v/v. The excitation wavelength was 345 nm. Dotted line: tubulin only; solid line: tubulin plus **4**; dashed line: tubulin plus **4** after addition of paclitaxel.

receptor site. Although compound **4** is not a high affinity ligand for this site, the micromolar dissociation constant should still be sufficient for competition binding assays in vitro. This possibility is illustrated with in Figure 5. Addition of paclitaxel to microtubules bound to probe **4** greatly decreased ligand fluorescence. These data also support the idea that, like the parent molecule discodermolide, the microtubule binding site for the fluorescent discodermolide overlaps with the paclitaxel site on microtubules.

We note that **4** is the second type of fluorescent probe based on discodermolide.[14] The earlier probes possess a dansyl group appended to the C-24 carbon by a spacer that contains 5 or 6 methylene groups. The activities of these molecules are either identical or very similar to discodermolide with respect to promotion of microtubule assembly and cytotoxicity. The high activity is to be expected since the bulky dansyl group is tethered to the parent molecule rather than included as an integral part of the structure. No fluorescence data have been reported for these probes. We speculate that the environment experienced by the sensitive dansyl group is primarily on the exterior of the receptor site.

In conclusion, a simplified fluorescently-labeled discodermolide analogue possessing a dimethylaminobenzoyl fluorophore was designed, synthesized, and biologically evaluated for antiproliferative activity, tubulin assembly activity, and microtubule binding. The molecule maintained some of the activity of the parent discodermolide, but to a diminished extent. The large increase in fluorescence intensity that occurs with microtubule binding is indicative of a hydrophobic local environment for the fluorophore in the discodermolide binding site on tubulin. This probe is suitable for direct observation of the discodermolide analogue binding to stabilized microtubules. It should be useful as donor molecules for fluorescence resonance energy transfer measurements and for assessing potential discodermolide-site microtubule stabilizers in vitro.

3. Experimental

3.1. General experimental methods

All chemicals obtained from commercial sources were used without further purification. The anhydrous reactions were performed under nitrogen or argon. All solvents were of reagent grade or HPLC grade. Tetrahydrofuran (THF) was distilled over sodium/benzophenone, and dichloromethane (DCM) was distilled over calcium hydride. The reactions were monitored by the analytical thin layer chromatography (TLC) plates (Silica Gel 60 GF) and analyzed with 254 nm UV light, $KMnO_4$ stain, and/or vanillin/sulfuric acid spray. Preparative thin layer chromatography (PTLC) plates were purchased from Analtech. All NMR spectral data were obtained on JEOL Eclipse spectrometer at 500 MHz, Varian Unity or Varian Inova spectrometer at 400 MHz. Chemical shifts reported as δ-values relative to known solvent residue peaks. High-resolution mass spectra (HRMS) were obtained in the analytical service in the Department of Chemistry at Virginia Tech. Known compounds were prepared through the reported procedures, and the NMR data of these compounds matched the literature values. All UV–vis spectroscopy was conducted in HP 8453 UV–vis spectrophotometer equipped with a multi-cell thermostated cuvette holder, and all fluorescence spectroscopy in a Jobin Yvon Fluorolog 3 spectrofluorometer in a thermostatted cell. All compounds were more than 95% pure as judged by TLC and ^{1}H NMR.

3.2. Synthesis

3.2.1. (2*R*,3*R*,4*S*,5*Z*,8*S*,9*R*,10*R*,11*S*,12*S*,*Z*)-9-(*tert*-Butyldimethylsilyloxy)-11-(4-methoxybenzyloxy)-3-(*O*-methoxymethyl)-2,4,6,8,10,12-hexamethylhexadec-5-enal (5)

To a solution of iodide **8**[16b] (87 mg, 0.15 mmol) in ether (1.74 mL) was added *t*-BuLi (0.18 mL, 1.7 M in pentane, 0.3 mmol), followed by MeO-9-BBN (0.3 mL, 1 M in hexane, 0.3 mmol) and THF (1.74 mL) at −78 °C. After stirring at rt for 1 h, Cs_2CO_3 solution (0.13 mL, 3 M in water, 0.38 mmol), a solution of vinyl iodide **9**[17d]

(67 mg, 0.15 mmol) in DMF (1.4 mL) and Pd(dppf)Cl_2 (6.2 mg) were added successively. The resulting solution was covered with aluminum foil and stirred at rt for 20 h. After dilution with water and ether, the mixture was extracted with ether, washed by brine, dried over Na_2SO_4, concentrated and purified by flash chromatography (5% EtOAc in hexanes) to afford TBS ether **10** (86 mg, 77%) as a colorless oil. $[\alpha]_D^{23}$ +4.0 (*c* 10.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.28 (2H, d, *J* = 7.2 Hz), 6.87 (2H, d, *J* = 8.3 Hz), 5.12 (1H, d, *J* = 10.2 Hz), 4.64 (2H, q, *J* = 6.5 Hz), 4.49 (2H, dd, *J* = 33.5, 10.7 Hz), 3.80 (3H, s), 3.64 (1H, dd, *J* = 10.0, 3.8 Hz), 3.52–3.47 (2H, m), 3.41 (3H, s), 3.21 (1H, t, *J* = 5.8 Hz), 3.14 (1H, t, *J* = 5.3 Hz), 2.64–2.49 (1H, m), 2.22 (1H, t, *J* = 13.3 Hz), 1.95–1.80 (3H, m), 1.80–1.73 (2H, m), 1.63 (3H, s), 1.45–1.23 (4H, m), 1.20–1.12 (2H, m), 0.99 (3H, d, *J* = 6.6 Hz), 0.96 (3H, d, *J* = 6.8 Hz), 0.95–0.87 (9H, m), 0.94 (9H, s), 0.89 (9H, s), 0.75 (3H, d, *J* = 6.7 Hz), 0.07 (3H, s), 0.06 (3H, s), 0.03 (6H, s).

TBS ether **10** (86 mg, 0.11 mmol) was dissolved in HCl/EtOH (1:99, 3.8 mL) at rt and stirred for 20 min. The reaction was quenched with $NaHCO_3$ and extracted with DCM, dried over Na_2SO_4, filtered, and concentrated to give the crude alcohol without purification. To the crude alcohol (50 mg, 0.077 mmol) in DCM (0.4 mL) and DMSO (0.4 mL) was added Et_3N (0.11 mL, 0.77 mmol) and pyridine sulfur trioxide complex (122 mg, 0.77 mmol) at 0 °C. After stirring at 0 °C for 1 h, NH_4Cl solution and water was added. The mixture was extracted with DCM, washed by water and brine, dried over Na_2SO_4, filtered, concentrated and purified by flash chromatography (15% EtOAc in hexanes) to afford aldehyde **5** (33 mg, 65% over 2 steps) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.62 (1H, d, *J* = 1.2 Hz), 7.27 (2H, d, *J* = 8.0 Hz), 6.88 (2H, d, *J* = 8.6 Hz), 4.85 (1H, d, *J* = 10.5 Hz), 4.67 (2H, dd, *J* = 8.2, 7.1 Hz), 4.53 (1H, d, *J* = 10.8 Hz), 4.43 (1H, d, *J* = 10.8 Hz), 3.80 (3H, s), 3.62 (1H, dd, *J* = 8.1, 4.0 Hz), 3.46 (1H, dd, *J* = 5.2, 3.5 Hz), 3.37 (3H, s), 3.13 (1H, t, *J* = 5.2 Hz), 2.69–2.59 (2H, m), 2.18 (1H, t, *J* = 12.4 Hz), 1.95–1.81 (2H, m), 1.77–1.65 (2H, m), 1.58–1.56 (3H, m), 1.52 (1H, ddd, *J* = 10.3, 7.6, 3.1 Hz), 1.45–1.23 (3H, m), 1.19–1.13 (2H, m), 1.09 (3H, d, *J* = 7.0 Hz), 1.01 (3H, d, *J* = 6.6 Hz), 0.98 (3H, d, *J* = 6.9 Hz), 0.96 (3H, d, *J* = 6.8 Hz), 0.93 (9H, s), 0.89 (3H, t, *J* = 7.2 Hz), 0.72 (3H, d, *J* = 6.7 Hz), 0.07 (3H, s), 0.06 (3H, s); ^{13}C NMR (500 MHz, $CDCl_3$) δ 202.7, 159.0, 135.7, 131.3, 129.1, 128.6, 113.7, 97.4, 85.3, 84.1, 77.5, 74.5, 56.0, 55.3, 49.5, 39.0, 37.3, 35.9, 35.2, 35.1, 31.1, 29.9, 26.3, 23.1, 18.5, 17.4, 17.2, 14.2, 13.5, 11.4, 9.9, −3.4, −3.4; HRMS (APCI+) calcd for $C_{38}H_{69}O_6Si$ $[M+H]^+$ 649.4863, found 649.4865.

3.2.2. 1-(Bis(2,2,2-trifluoroethyl)phosphoryl)-4-(*tert*-butyldimethylsilyloxy)butan-2-one (6)

To a solution of 1,3-propanediol (3 mL, 40 mmol) in THF (60 mL) was added NaH (1.2 g, 60%, 20 mmol) at rt. After stirring for 2.5 h, TBSCl (630 mg, 4.3 mmol) was added and the mixture was stirred overnight before the addition of $NaHCO_3$ solution in MeOH. The solution was then extracted with DCM, washed with water and brine, dried over Na_2SO_4, filtered, concentrated and purified by flash chromatography (30% EtOAc in hexanes) to afford 2-((*tert*-butyldimethylsilyl)oxy)ethanol ether (800 mg, 90% based on TBSCl) as a colorless oil. To a solution of this TBS ether (590 mg, 3.11 mmol) in DCM (29 mL) was added $NaHCO_3$ and Dess–Martin periodinane at rt. After stirring for 30 min, the solution was concentrated, diluted with hexane to participate byproduct, filtered and concentrated carefully to give crude 2-((*tert*-butyldimethylsilyl)oxy)acetaldehyde. A solution of this crude aldehyde in *t*-BuOH (13 mL) and 2-methyl-2-butene (1.5 mL) was treated with a solution of $NaClO_2$ (500 mg, 5.5 mmol) and NaH_2PO_4 (1.85 g, 15.4 mmol) in water (12 mL) dropwise. After stirring for 1 h, the reaction was partitioned between brine and DCM. The organic layer was dried over Na_2SO_4, filtered, concentrated and purified by flash chromatography (30% EtOAc in hexanes) to afford acid **11** (431 mg, 68% over 2 steps) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.91 (2H, t, *J* = 6.1 Hz), 2.58 (2H, t, *J* = 6.1 Hz), 0.89 (9H, s), 0.09 (6H, s).

To a solution of acid **11** (166 mg, 0.81 mmol) in DCM (9 mL) was added 1-chloro-*N*,*N*,2-trimethyl-1-propenylamine (0.24 mL, 1.8 mmol). After stirring for 1 h, the solution was concentrated and the crude acid chloride **12** was dried in vacuo for 30 min. Meanwhile, LiHMDS (2.7 mL, 1.0 M in THF, 2.7 mmol) was added dropwise to a solution of methylphosphonic acid bis(2,2,2-trifluoroethyl) ester (700 mg, 2.67 mmol) in THF (2 mL) at −98 °C. After stirring for 15 min, a solution of the crude acid chloride **12** in THF (5.8 mL) was added at −98 °C. The resulting solution was stirred for 1 h at −98 °C before adding NH_4Cl solution to quench the reaction. The solution was then warmed to rt, and more NH_4Cl solution was added. The mixture was extracted with DCM, dried over Na_2SO_4, filtered, concentrated and purified by flash chromatography (30% EtOAc in hexanes) to afford phosphonate **6** (135 mg, 40% over 2 steps) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.50–4.39 (4H, m), 3.90 (2H, t, *J* = 6.0 Hz), 3.38 (1H, s), 3.34 (1H, s), 2.74 (2H, td, *J* = 6.0, 0.8 Hz), 0.88 (9H, s), 0.06 (6H, s); ^{13}C NMR (500 MHz, $CDCl_3$) δ 200.7, 122.6 (dd, *J* = 551.5, 16.6 Hz), 62.5 (dq, *J* = 75.8, 11.1 Hz), 58.6, 47.2 (d, *J* = 10.4 Hz), 42.8, 41.7, 25.9, 18.3, −5.5; HRMS (ESI+) calcd for $C_{14}H_{26}F_6O_5PSi$ $[M+H]^+$ 447.1191, found 447.1186.

3.2.3. (8*Z*,10*S*,11*S*,12*S*,13*Z*,16*S*,17*R*)-17-((2*R*,3*S*,4*S*)-3-(4-Methoxybenzyloxy)-4-methyloctan-2-yl)-11-(*O*-methoxymethyl)-2,2,3,3,10,12,14,16,19,19,20,20-dodecamethyl-4,18-dioxa-3,19-disilahenicosa-8,13-dien-7-one (13)

K_2CO_3 (60 mg, 0.43 mmol) and 18-crown-6 (228 mg, 0.86 mmol) were dissolved in toluene (0.6 mL) and HMPA (0.06 mL) at rt and the mixture was stirred for 3 h before adding a solution of aldehyde **5** (38 mg, 0.07 mmol) and phosphonate **6** (103 mg, 0.23 mmol) in toluene (0.6 mL) and HMPA (0.06 mL) at −10 °C. After stirring at −5 °C for 18 h, NH_4Cl solution was added to quench the reaction and the mixture was extracted with EtOAc, washed with brine, dried over Na_2SO_4, filtered, concentrated and purified by flash chromatography (20% EtOAc in hexanes) to afford the new TBS ether **13** (51 mg, 85%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.27 (2H, d, *J* = 8.5 Hz), 6.87 (2H, d, *J* = 8.7 Hz), 6.15–6.08 (2H, m), 4.90 (1H, d, *J* = 10.3 Hz), 4.73 (2H, dd, *J* = 36.7, 6.7 Hz), 4.48 (2H, dd, *J* = 36.0, 10.8 Hz), 3.95–3.84 (2H, m), 3.80 (3H, s), 3.75–3.64 (1H, m), 3.49–3.46 (1H, m), 3.41 (3H, s), 3.25 (1H, d, *J* = 7.3, 3.8 Hz), 3.13 (1H, t, *J* = 5.4 Hz), 2.73–2.55 (2H, m), 2.54–2.42 (1H, m), 2.16 (1H, t, *J* = 12.5 Hz), 1.95–1.80 (2H, m), 1.74–1.65 (2H, m), 1.57 (3H, s), 1.51 (1H, ddd, *J* = 10.7, 8.0, 2.7 Hz), 1.45–1.25 (3H, m), 1.20–1.13 (2H, m), 1.02 (3H, d, *J* = 6.9 Hz), 0.98 (3H, d, *J* = 6.9 Hz), 0.96 (3H, d, *J* = 6.8 Hz), 0.96 (3H, d, *J* = 6.6 Hz), 0.93 (9H, s), 0.89 (3H, t, *J* = 7.3 Hz), 0.87 (9H, s), 0.72 (2H, d, *J* = 6.7 Hz), 0.07 (3H, s), 0.06 (3H, s), 0.04 (3H, s), 0.04 (3H, s); ^{13}C NMR (500 MHz, $CDCl_3$) δ 199.6, 159.0, 151.1, 133.0, 131.4, 130.0, 129.0, 125.7, 113.7, 97.6, 86.9, 85.6, 77.5, 74.6, 59.1, 56.2, 55.3, 47.2, 38.8, 36.7, 36.2, 35.9, 35.6, 35.4, 30.9, 29.9, 29.7, 26.3, 25.9, 23.1, 18.5, 18.3, 17.5, 17.3, 16.9, 14.2, 14.0, 11.4, −3.4, −3.5, −5.4, −5.4; HRMS (ESI+) calcd for $C_{48}H_{88}O_7Si_2Na$ $[M+Na]^+$ 855.5966, found 855.5944.

3.2.4. (8*Z*,10*S*,11*S*,12*S*,13*Z*,16*S*,17*R*)-17-((2*R*,3*S*,4*S*)-3-Hydroxy-4-methyloctan-2-yl)-11-(methoxymethoxy)-2,2,3,3,10,12,14,16,19,19,20,20-dodecamethyl-4,18-dioxa-3,19-disilahenicosa-8,13-dien-7-one (14)

To a vigorously stirred solution of PMB ether **13** (45 mg, 0.06 mmol) in DCM (2.7 mL) and pH 7 buffer (0.3 mL) was added DDQ (25 mg, 0.11 mmol) at 0 °C. After stirring for 1.5 h, $NaHCO_3$ solution was added and the mixture was extracted with DCM, washed by $Na_2S_2O_3$ solution, water and brine, dried over Na_2SO_4,

filtered, concentrated and purified by flash chromatography (20% EtOAc in hexanes) to give alcohol **14** (28 mg, 75%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.21–6.08 (2H, m), 4.92 (1H, d, J = 10.2 Hz), 4.73 (2H, dd, J = 24.2, 6.7 Hz), 3.90 (2H, qt, J = 10.3, 6.6 Hz), 3.71 (1H, ddd, J = 9.0, 6.9, 3.7 Hz), 3.65 (1H, t, J = 3.9 Hz), 3.41 (3H, s), 3.26 (2H, dd, J = 7.2, 3.8 Hz), 2.70 (1H, dt, J = 15.9, 6.7 Hz), 2.60 (1H, dt, J = 15.9, 6.4 Hz), 2.54–2.45 (1H, m), 2.14 (1H, t, J = 12.1 Hz), 1.90–1.79 (3H, m), 1.70 (1H, br s), 1.66–1.61 (1H, m), 1.59 (3H, d, J = 0.9 Hz), 1.55–1.50 (1H, m), 1.43–1.26 (3H, m), 1.24–1.15 (1H, m), 1.13–1.05 (1H, m), 1.03 (3H, d, J = 6.9 Hz), 0.97 (3H, d, J = 6.6 Hz), 0.92 (9H, s), 0.90 (3H, t, J = 7.3 Hz), 0.89 (3H, d, J = 7.0 Hz), 0.87 (9H, s), 0.84 (3H, d, J = 6.7 Hz), 0.74 (3H, d, J = 6.9 Hz), 0.10 (3H, s), 0.10 (3H, s), 0.05 (3H, s), 0.04 (3H, s); ^{13}C NMR (500 MHz, $CDCl_3$) δ 199.6, 151.0, 133.3, 130.0, 125.8, 97.8, 87.1, 79.7, 79.1, 59.0, 56.2, 47.2, 36.9, 36.4, 36.2, 36.0, 35.8, 35.3, 31.9, 29.2, 26.2, 25.9, 23.2, 23.2, 18.4, 18.3, 17.5, 17.1, 16.0, 14.5, 14.2, 8.8, −3.2, −4.1, −5.4, −5.4; HRMS (ESI+) calcd for $C_{40}H_{80}O_6Si_2Na$ $[M+Na]^+$ 735.5391, found 735.5401.

3.2.5. (5*S*,6*S*,7*R*,8*R*,9*S*,11*Z*,13*S*,14*S*,15*S*,16*Z*)-8,20-Bis(*tert*-butyldimethylsilyloxy)-14-(methoxymethoxy)-5,7,9,11,13,15-hexamethyl-18-oxoicosa-11,16-dien-6-yl carbamate (15)

To a solution of alcohol **14** (28 mg, 0.04 mmol) in DCM (3.7 mL) was added $Cl_3CCONCO$ (24 μL, 0.2 mmol) at rt. After stirring for 1.5 h, the solution was loaded onto a neutral Al_2O_3 plug and left for 2 h. The Al_2O_3 plug was then flushed with EtOAc (100 mL) and the solution was concentrated and purified by flash chromatography (30% EtOAc in hexanes) to afford carbamate **15** (24 mg, 82%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.17–6.06 (2H, m), 4.86 (1H, d, J = 10.3 Hz), 4.73 (2H, dd, J = 33.8, 6.7 Hz), 4.69 (2H, br s), 4.61 (1H, dd, J = 7.2, 4.6 Hz), 3.91 (2H, t, J = 6.8 Hz), 3.68 (1H, dqd, J = 10.3, 6.9, 3.6 Hz), 3.40 (3H, s), 3.40–3.38 (1H, m), 3.22 (1H, dd, J = 7.8, 3.5 Hz), 2.67 (2H, td, J = 6.8, 2.7 Hz), 2.50–2.41 (1H, m), 2.15 (1H, t, J = 12.4 Hz), 1.96–1.87 (2H, m), 1.73–1.62 (2H, m), 1.59 (3H, s), 1.45–1.23 (4H, m), 1.17–1.03 (2H, m), 1.01 (3H, d, J = 7.0 Hz), 0.94 (3H, d, J = 6.6 Hz), 0.91 (9H, s), 0.90–0.85 (9H, m), 0.86 (9H, s), 0.69 (3H, d, J = 6.8 Hz), 0.07 (3H, s), 0.06 (3H, s), 0.04 (3H, s), 0.04 (3H, s); ^{13}C NMR (500 MHz, $CDCl_3$) δ 199.4, 157.1, 151.1, 133.4, 129.6, 125.7, 97.8, 87.2, 79.9, 77.1, 59.1, 56.2, 47.1, 37.4, 36.6, 36.3, 36.0, 34.9, 34.9, 31.1, 29.4, 26.2, 25.9, 23.0, 22.9, 18.5, 18.3, 17.7, 17.0, 16.1, 14.1, 13.0, 10.5, −3.4, −3.6, −5.4; HRMS (ESI+) calcd for $C_{41}H_{81}NO_7Si_2Na$ $[M+Na]^+$ 778.5449, found 778.5462.

3.2.6. (5*S*,6*S*,7*R*,8*R*,9*S*,11*Z*,13*S*,14*S*,15*S*,16*Z*,18*S*)-8,20-Bis(*tert*-butyldimethylsilyloxy)-18-hydroxy-14-(*O*-methoxymethyl)-5,7,9,11,13,15-hexamethylicosa-11,16-dien-6-yl carbamate (16)

A solution of ketone **15** (15 mg, 0.02 mmol) in toluene (3.7 mL) was treated with K-selectride™ (40 μL, 1 M in THF, 0.04 mmol) at −78 °C. After stirring for 2 h, one drop of AcOH was added to quench the reaction. The solution was diluted with EtOAc, washed with water and brine, dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash chromatography (15% EtOAc in hexanes) to give alcohol **16** (14 mg, 90%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.49–5.38 (2H, m), 5.03 (1H, d, J = 10.1 Hz), 4.63 (2H, s), 4.62–4.54 (3H, m), 3.91 (1H, dt, J = 10.3, 5.3 Hz), 3.88–3.81 (1H, m), 3.43 (1H, dd, J = 5.9, 3.0 Hz), 3.39 (3H, s), 3.14–3.07 (1H, m), 2.81 (1H, dd, J = 13.8, 6.8 Hz), 2.58–2.50 (1H, m), 2.19 (1H, t, J = 12.3 Hz), 1.98–1.90 (2H, m), 1.79–1.66 (4H, m), 1.64 (3H, s), 1.44–1.27 (4H, m), 1.19–1.04 (2H, m), 1.02 (3H, d, J = 6.9 Hz), 0.95 (3H, d, J = 6.7 Hz), 0.92 (9H, s), 0.90 (9H, s), 0.90–0.85 (9H, m), 0.73 (3H, d, J = 6.8 Hz), 0.08 (3H, s), 0.07 (3H, s), 0.07 (3H, s), 0.06 (3H, s); ^{13}C NMR (500 MHz, $CDCl_3$) δ 157.1, 134.3, 132.9, 131.8, 130.3, 98.2, 87.3, 80.0, 77.2, 68.2, 62.4, 56.1, 38.8, 37.3, 36.4, 35.7, 35.1, 35.0, 31.1, 29.7, 29.4, 26.2, 25.9, 23.1, 23.0, 18.5, 18.3, 18.2, 16.6, 16.1, 14.1, 13.1, 10.5, −3.4, −3.7, −5.5; HRMS (ESI+) calcd for $C_{41}H_{83}NO_7Si_2Na$ $[M+Na]^+$ 780.5606, found 780.5615.

3.2.7. (3*S*,4*Z*,6*S*,7*S*,8*S*,9*Z*,12*S*,13*R*,14*S*,15*S*,16*S*)-15-(Carbamoyloxy)-3,7,13-trihydroxy-6,8,10,12,14,16-hexamethylicosa-4,9-dienyl 3-(dimethylamino)benzoate (4)

TBS ether **16** (18 mg, 0.024 mmol) was dissolved in MeOH (3.6 mL). After stirring for 15 min, HCl solution (3.6 mL, 4 M in water) was added to the solution in small portions (0.15 mL per 10 min in 4 h) at rt before $NaHCO_3$ was added carefully to quench reaction. The solution was extracted with EtOAc, washed with brine, dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash chromatography (80% EtOAc in hexanes) to give alcohol **17** (10 mg, 88%) as a colorless oil.

To a solution of 3-(dimethylamino)benzoic acid (2.8 mg, 17 μmol) and DMAP (1.5 mg, 12.3 μmol) in DCM (0.6 mL) was added a solution of alcohol **17** (4.2 mg, 8.7 μmol) in DCM (0.6 mL), followed by EDCI (3.9 mg, 20 μmol). After stirring at rt for 12 h, water was added and the mixture was extracted with EtOAc. The organic layer was then washed by brine, dried over Na_2SO_4, filtered, concentrated and purified by flash chromatography (50% EtOAc in hexanes) and to give **4** (1 mg, 18%) as a colorless oil. $[\alpha]_D^{25}$ −12.5 (*c* 0.2, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.40 (1H, dd, J = 2.6, 1.4 Hz), 7.35 (1H, dd, J = 7.6, 1.2 Hz), 7.29 (1H, t, J = 7.9 Hz), 7.00 (1H, ddd, J = 8.2, 2.7, 0.9 Hz), 5.60 (1H, d, J = 10.3 Hz), 5.42 (1H, dd, J = 10.7, 9.2 Hz), 5.03 (1H, d, J = 10.0 Hz), 4.65 (1H, t, J = 5.9 Hz), 4.53 (1H, td, J = 8.6, 3.6 Hz), 4.42 (2H, dd, J = 7.3, 5.6 Hz), 3.20 (1H, t, J = 5.7 Hz), 3.14 (1H, dd, J = 7.7, 3.8 Hz), 2.99 (6H, s), 2.72–2.67 (1H, m), 2.52–2.37 (2H, m), 2.18–2.08 (2H, m), 1.97–1.89 (3H, m), 1.88–1.78 (2H, m), 1.74 (1H, dd, J = 11.6, 2.6 Hz), 1.59 (3H, s), 1.46–1.41 (2H, m), 1.22–1.11 (4H, m), 1.07 (3H, d, J = 7.0 Hz), 0.95 (3H, d, J = 6.6 Hz), 0.93 (3H, t, J = 7.2 Hz), 0.91–0.89 (6H, m), 0.78 (3H, d, J = 6.6 Hz); ^{13}C NMR (500 MHz, $CDCl_3$) δ 167.6, 159.1, 150.8, 133.0, 132.9, 132.1, 130.7, 130.2, 128.8, 117.4, 117.0, 112.9, 79.2, 76.4, 64.1, 61.6, 48.3, 39.5, 36.8, 36.5, 36.3, 36.1, 35.6, 34.4, 32.8, 30.4, 29.1, 22.7, 22.2, 18.2, 16.4, 15.3, 13.2, 13.0, 8.3; HRMS (ESI+) calcd for $C_{36}H_{60}N_2O_7Na$ $[M+Na]^+$ 655.4298, found 655.4306.

3.3. Biological experiments

3.3.1. Handling and storage

Stock solutions of analogue **4** were made in DMSO and stored at −20 °C until use. Concentrations were determined spectrophotometrically using a molar absorptivity of $\varepsilon_{345\ nm}$ = 1800 M^{-1} cm^{-1} as determined in the SUNY laboratory.

3.3.2. Antiproliferative activities

Antiproliferative activity against the A2780 cell line[24] was determined as described previously.

3.3.3. Tubulin isolation

Tubulin was isolated from bovine brains by two cycles of temperature-dependent polymerization and depolymerization followed by phosphocellulose ion-exchange chromatography.[25] The protein containing fractions were then pooled, drop frozen, and stored on liquid nitrogen until use. Prior to use, frozen aliquots were gently thawed and desalted into PME buffer (0.1 M Pipes, 1 mM $MgSO_4$, 2 mM EDTA, pH 6.90) The concentration was determined spectrophotometrically using an extinction coefficient of 1.23 $(mg/mL)^{-1}$ cm^{-1} at 278 nm.[26]

3.3.4. EC_{50} measurement

The EC_{50} is defined as the concentration of the molecule required to assemble the protein to 50% of the activity at saturation. This was quantified for the discodermolide analogues in reference

to the standard paclitaxel. Analogue **4** was evaluated by light scattering.[27] Samples containing 5 μM soluble tubulin in PME with 0.1 mM GTP were equilibrated to 37 °C and polymerization induced by addition of varying concentrations of drug dissolved in DMSO to a final concentration of 10% v/v. Polymerization activity was monitored as apparent absorption increase at 350 nm, taking the plateau value with the baseline subtracted to be polymerization extent. The plateau value was graphed on a linear axis as a function of drug concentration and the EC_{50} value extracted from a square hyperbolic fit in SigmaPlot 10.0 (Systat Software Inc., San Jose, CA).

3.3.5. Microtubule binding properties

Because the analogue showed a fluorescence increase upon binding to tubulin, the increase in fluorescence at 440 nm was used to measure binding. Dissociation constants were assessed by titration of ligand fluorescence at 25 °C using a fixed concentration of ligand and varying concentrations of the receptor, GMPcPP microtubules (prepared as described by Vulevic and Correia)[28] in PME buffer containing 0.1 mM GTP and 10% DMSO. The data were fit to the equation

$$f = F_{max} * [MT]/(K_d + [MT])$$

where f is the fluorescence intensity at the emission maximum of the ligand at each concentration, F_{max} is the maximum fluorescence, [MT] is the total concentration of tubulin in GMPcPP-microtubules, and K_d is the dissociation constant. Saturation was not achieved for compound **4**; therefore the value for F_{max} was obtained from double reciprocal plots. At the end of the titration, competition by paclitaxel was assessed in the following manner: paclitaxel in DMSO was added to a total concentration equal to the concentration of tubulin and a fluorescence spectrum was taken.

Competition with paclitaxel was also assessed with GTP-tubulin. Tubulin (10 μM) in PME buffer containing 1 mM GTP was equilibrated to 37 °C in the spectrofluorometer and an emission spectrum was collected. Excess probe in DMSO was added to a total concentration of 90 μM and the sample was incubated for an additional 30 min. After collection of the emission spectrum, paclitaxel in DMSO was added to a final concentration of 10 μM and 4% DMSO. The emission spectrum was collected 5 min after the addition of paclitaxel. The excitation wavelength was 345 nm.

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