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Synthesis and biological activities of high affinity taxane-based fluorescent probes

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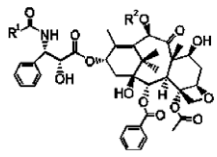
ABSTRACT

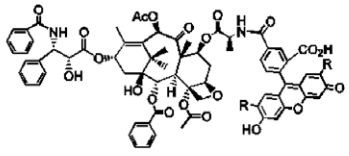
Three fluorescent probes **3a, 3b**, and **4** have been synthesized through conjugation of fluorescein and difluorescein groups to the 7-OH of C-2 modified paclitaxel and cephalomannine derivatives with very high affinity to microtubules. All these probes exhibited potent tubulin assembly promotion and tumor cell killing activities, thus may be useful as tools for the determination of thermodynamic parameters and exploration of ligand–microtubule interactions.

The plant-derived natural product paclitaxel (**1a**), exerting its mechanism through disruption of dynamics of tubulin/microtubule polymerization–depolymerization process,[1] has been widely used for the treatment of various solid tumors.[2] In order to study the interaction of **1a** with tubulin/microtubule system and visualize the microtubule dynamics in the presence of **1a**, various spectroscopic methods have been applied. One of the most useful techniques to explore such interactions is fluorescence spectroscopy,[3–5] in which various probes derived from **1a** or its derivatives with fluorophore tags at different positions of taxanes are often used.[6–12]

Most of taxane-based fluorescent probes are prepared from **1a**, by the introduction of a fluorophore at C-7 position through proper linkage. Because C-7 and other "northern hemisphere" substituents in **1a** are known not to interact with tubulin significantly, so these probes are believed to minimize the interference of large fluorescent groups such as fluorescein and rhodamine in the binding pocket. With the fluorescein and difluorescein-tagged **1a**, Flutax-1 (**2a**), and -2 (**2b**), it is possible to determine the thermodynamic and kinetic parameters for the taxane–tubulin binding process,[13–15] analyze the ligand's conformation in D_2O/DMSO-d_6,[16] and locate the centrosome and spindle pole microtubules as the main targets for taxanes to induce cell death.[11]



1a R^1=Ph, R^2=Ac
1b R^1=*t*-BuO, R^2=H



2a R=H
2b R=F

It has been found that precise determinations of thermodynamic parameters for ligand–tubulin binding is critical to quantify the enthalpic and entropic contributions to the binding process, thus predict the cytotoxicity of the ligands and help the design of high affinity ligands (i.e. taxanes) through the binding affinity dissection.[17–19] During the process of the determination of thermodynamic parameters by the competitive displacement method, fluorescent probes with binding affinity within three orders of magnitudes to the targeted ligands are usually applied. However, displacement methods cannot distinguish ligands with affinities much higher than probes (e.g. **2a** or **2b** in our case) thus it is not possible to determine the binding affinity or thermodynamic parameters precisely.[20] The thermodynamic parameter of binding of compounds with high affinity for the taxane site have been determined by displacement of a non-fluorescent compound, Epothilone B, with high affinity (K_a 7.5×10^8 M^{-1} at 35 °C) for the paclitaxel site.[17,19] In this method, the Epothilone B concentrations in supernatants and pellets required to measure the binding constant of the

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competitor have to be determined employing HPLC, thus the method has a low throughput and is time consuming. Hence, a high affinity fluorescent taxane is desirable for the parameter determination by fluorescence spectroscopy during the optimization of the binding affinity of taxanes to tubulin.

The introduction of methoxy or azido group to the *meta* position of C-2 benzoate phenyl in **1a** and its analogs has been proved to enhance the tubulin polymerization promotion ability and cytotoxicity significantly.[22,23] It was also known that the binding affinity of taxanes to tubulin/microtubule can be potentiated by such a modification.[15,19] So, we prepared fluorescent probes **3** and **4** from 2-(*m*-MeO)-benzoyl paclitaxel and 2-(*m*-N_3)-benzoyl cephalomannine which are higher affinity tubulin binders than **1** and **2**, and investigated their fluorescence properties and biological activities.

Chemical syntheses. The synthesis of **3** was performed in a similar way to that of 2-modified taxanes,[19,21] except the 7-TES group was replaced with 7-alanine (7-Ala).

Paclitaxel (**1a**) was first protected at 2′-OH and then conjugated with Boc-Ala-OH at 7-OH to afford **5**. Compound **5**, upon 2-debenzoylation and subsequent acylation, was converted to 2-(*m*-MeO-Bz)-paclitaxel analog **6**. Deprotection of 2′-*O*-*tert*-butyldimethylsilyl and *N*-*tert*-butyloxycarbonyl groups under mild conditions furnished 7-Ala-2-modified paclitaxel analog **7**, with free amino group ready for the conjugation of fluorescent groups. Coupling of **7** with 5-fluorescein succinimidyl ester (5-FAM SE) or 5-difluorescein succinimidyl ester (OG 488 SE) in sodium carbonate–dicarbonate buffer (pH 9.0) furnished the desired conjugates **3a** (20% yield) and **3b** (60% yield) (Scheme 1).

The fluorescent probe **4** was also prepared from cephalomannine similarly in 14% overall yield for seven steps.

The final products **3**[25,26] and **4**,[27] after chromatographic separations, are still contaminated by ca. 20–55% weight of NaOAc (ca. 1:20–1:4 taxane–NaOAc peak ratios in the corresponding NMR spectra). Since these impurities do not interfere with the parameter determination, the probes are not subjected to further purifications.

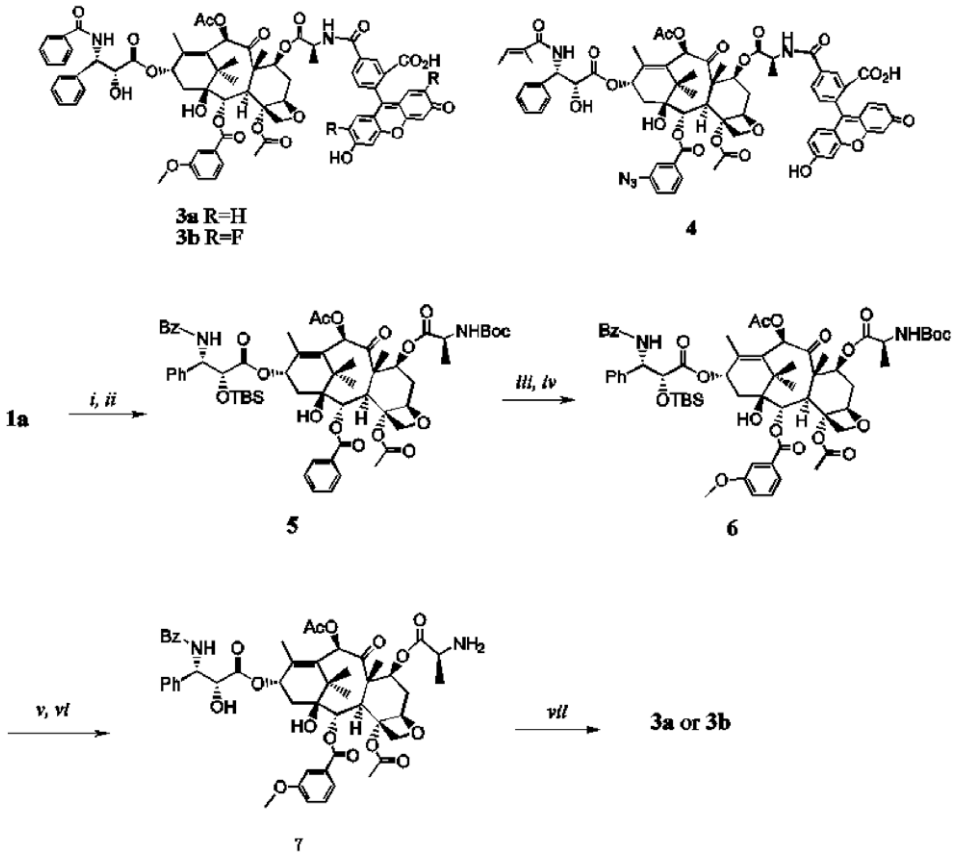
Binding to the paclitaxel site in microtubules. The compounds were tested for their ability to bind tubulin in the paclitaxel site. All of them, upon binding to the paclitaxel site, underwent a large increase in the anisotropy of their fluorescence as is the case for other fluorescent derivatives of paclitaxel[13] (Table 1).

As expected for the introduction of binding enhancement groups[15,19] in **1a**, docetaxel (**1b**) does not displace them from the crosslinked microtubules (no decrease in the anisotropy of binding is observed after addition of 10 μM docetaxel) as in the case for

Table 1
Anisotropy of the fluorescence emission of the fluorescent taxoids in different status[a]

Ligands	Free	Bound[b]	Displaced[b]
2a[c]	0.05 ± 0.02	0.24 ± 0.02	0.05 ± 0.02
2b[c]	0.04 ± 0.01	0.26 ± 0.02	0.08 ± 0.01
3a	0.046 ± 0.003	0.168 ± 0.007	0.052 ± 0.008
3b	0.046 ± 0.001	0.202 ± 0.031	0.068 ± 0.010
4	0.059 ± 0.010	0.291 ± 0.020	0.077 ± 0.010

[a] Determined with fluorescent ligands (50 nM) in GAB buffer at 25 °C. Excitation and emission wavelengths for **3a** and **3b** are 492 and 524 nm, and for **4** 493 and 521 nm, respectively.
[b] Bound to 1 μM of paclitaxel binding sites in stabilized microtubules by crosslinking, and further displaced by the addition of 10 μM discodermolide.
[c] Data from Ref. 13. Bound to microtubules assembled from 20 μM pure tubulin in GAB, and further displaced by the addition of 50 μM docetaxel.



Scheme 1. Reagents and conditions: (i) TBSCl, imidazole, DMF, 70 °C, 97%; (ii) Boc-Ala-OH, DCC, DMAP, toluene, 35 °C, 86%; (iii) Triton B, CH_2Cl_2, −20 °C, 77%; (iv) *m*-methoxy benzoic acid, DCC, 4-PP, 65 °C, 73%; (v) 40% aq HF, Py, CH_3CN, 35 °C, 97%; (vi) CF_3COOH, $PhSCH_3$, CH_2Cl_2, 0 °C, 61%; (vii) 5-FAM SE or OG 488 SE, DMF, sodium carbonate–dicarbonate buffer (pH 9.0), rt, 20% for **3a**, 60% for **3b**.

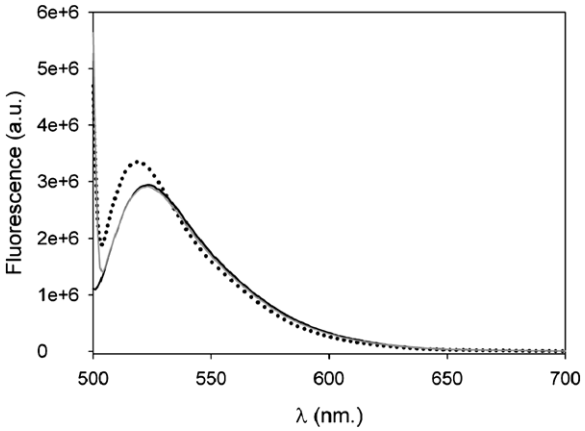


Figure 1. Fluorescence spectra of **4** (50 nM) black line, **4** plus microtubules (1 μM), dotted line and **4**, microtubules plus discodermolide (10 μM), gray line in GAB buffer.

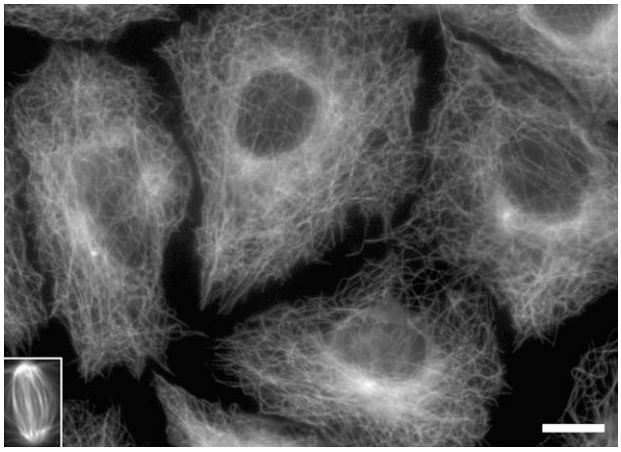


Figure 2. Visualization of microtubules with **4** in A549 human lung carcinoma cells. A549 unfixed microtubule cytoskeletons were incubated with 1 μM **4** for 5 min. Inset: **4** stained mitotic spindle from the same preparations. Scale bar represents 10 μm for both images.

Flutax-1 (**2a**) and Flutax-2 (**2b**).[13] Their binding constants to microtubules were also measured, and found to be $4.4 \pm 1.9 \times 10^8$, $6.8 \pm 1.7 \times 10^8$, and $12 \pm 4 \times 10^8$ M^{-1} for **3a, 3b**, and **4**, respectively. All these facts indicate that their binding affinities are much higher than that of docetaxel. The specificity of the binding was checked by adding 10 μM discodermolide, the natural paclitaxel binding site ligand with the highest affinity known to date, to the samples containing the fluorescent taxanes bound to stabilized microtubules. Discodermolide displaces the bound taxane, resulting in a decrease of the anisotropy of the fluorescence of the sample to the values observed for the free compound. The same effect can be shown by fluorescence intensity (Fig. 1). Addition of microtubules to the solution of **4** led to the change of its fluorescence spectra, with a small increase in fluorescence intensity, while addition of discodermolide to this solution restored the spectra, due to the displacement of fluorescent probe 4 from its binding site in microtubule.

Microtubule assembly induction. The compounds were then tested for their ability to induce microtubule assembly in conditions which tubulin do not assemble.[24] The compounds were able to induce assembly of GTP-tubulin into microtubules, indicating that the introduction of the fluorescent group does not perturb their tubulin promotion activity (for the critical concentration data, see Table 2). All three high affinity taxane-based fluorescent probes were found to be tubulin assembly promoters comparable with the more potent taxanes (including the clinically used taxanes, paclitaxel, and docetaxel, as well as two fluorescent taxanes (**2a** and **2b**) previously employed[12,20] (supplementary information of Ref. 19). Among the three probes, **3b** is the more active one, although the differences are in within the experimental errors of the docetaxel measurement.

Ligand binding to cellular microtubules and cytotoxicity. To test if these fluorescent probes can bind to native cellular microtubules, we incubated unfixed A549 human lung carcinoma cytoskeletons with them and imaged by fluorescence microscopy as with other fluorescent taxanes[12] (Fig. 2). It was found that they readily and brightly stained interphasic cellular microtubules as well as mitotic spindles, centrosomes and the central part of the midbody in cytokinesis.

Cytotoxicities were tested on A2780 ovarian carcinoma cells and their P-gp overexpressing counterpart A2780AD cells. All three newly synthesized compounds were able to kill the non-resistant cells (Table 3). However, despite having higher affinity to microtubules than paclitaxel (they cannot be displaced from crosslinked stabilized microtubules by a 200 times excess of docetaxel), their cytotoxicity is much lower than paclitaxel and docetaxel (Table 3) and their unlabeled partners,[19] indicating that the polar fluorescent moiety impedes their cell permeability. The poor cytotoxicity for **4**, **2a**, and **2b** in P-glycoprotein overexpressing A2780AD cells (which is not the case for their unlabeled partners[19]) may suggest that the introduction of the fluorescent moiety usually makes them better substrates for membrane transporter proteins such as P-glycoprotein. However, Fchitax-2 (**3a**) and Fchitax-3 (**3b**) are more active in resistant cells (*R*/*S* index of 36 and 26, respectively), than their parent compound Chitax-11 (*R*/*S* index 120). It is also interesting to note that cytotoxicities of the difluorescein bearing probes (**2b and 3b**) are less active than fluorescein bearing ones (**2a and 3a**), although the former are slightly more potent tubulin polymerization promoters.

In conclusion, all three fluorescent probes can be applied to the determination of binding affinity or thermodynamic parameters for taxanes with much higher affinity than **1**. In addition, **3a** and

Table 2
Critical concentrations of the ligand-induced assembly reactions[a]

Ligands	Critical concentrations (μM)	Ligands	Critical concentrations (μM)
4	2.45 ± 0.41	**1a**[b]	4.23 ± 0.68
3a	2.26 ± 0.40	**1b**[b]	2.50 ± 0.37
3b	2.17 ± 0.39	**2a**	3.84 ± 0.56
		2b	3.22 ± 0.46

[a] Determined in the presence of a 10% excess of ligand in 10 mM phosphate, 1 mM EDTA, 4 mM $MgCl_2$, pH 6.7 solution in the presence of 1 mM GTP.[17]
[b] Data from Ref. 17.

Table 3
Cytotoxicity of the compounds against drug-sensitive and -resistant cells

Ligands	A2780[a] (nM)	A2780AD	*R*/*S*[b]
4	220 ± 15	>20,000	>91
3a	19.5 ± 0.35	716 ± 120	36.7
3b	272 ± 12	7200 ± 200	26.4
1a	1.3 ± 0.4	980 ± 149	753
1b	0.6 ± 0.2	290 ± 16	483
2a	256 ± 17	>20,000	>78
2b	800 ± 28	>20,000	>25

[a] IC_{50} values were determined in parental ovarian carcinoma A2780 cells and its drug resistant counterpart A2780AD cells after 48 h incubation in the presence of the drugs using an MTT cell proliferation assay.[15]
[b] The resistance factor is calculated dividing the IC_{50} of the resistant cell line by the IC_{50} of the drug sensitive cell line.

3b may also be useful in the exploration of ligand–microtubule interactions at cellular level.

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25. *Spectral data for* ***3a***. ^{1}H NMR (500 MHz, CD_3OD): δ 8.41 (1H, d, J = 2.0 Hz), 7.96 (1H, dd, J = 1.5, 7.5 Hz), 7.80 (2H, m), 7.62 (1H, m), 7.59 (1H, m), 7.50–7.16 (11H, m), 6.96 (2H, dd, J = 2.0, 9.0 Hz), 6.47 (2H, dd, J = 2.0, 9.0 Hz), 6.44 (2H, m), 6.31 (1H, s), 6.09 (1H, t, J = 9.0 Hz), 5.62 (1H, dd, J = 7.5, 11.0 Hz), 5.60 (1H, d, J = 7.0 Hz), 5.56 (1H, d, J = 5.5 Hz), 4.96 (1H, d, J = 10.0 Hz), 4.71 (1H, d, J = 6.0 Hz), 4.59 (1H, q, J = 7.5 Hz), 4.20 (1H, d, J = 8.5 Hz), 4.14 (1H, d, J = 8.0 Hz), 3.87 (1H, d, J = 7.0 Hz), 3.85 (3H, s), 2.56 (1 H, ddd, J = 5.5, 8.0, 15.0 Hz), 2.30 (3H, s), 2.17 (1H, dd, J = 9.5, 15.5 Hz), 2.13 (3H, s), 1.93 (1H, dd, J = 9.5, 15.5 Hz), 1.86 (3H, s), 1.79 (1H, overlapped), 1.76 (3H, s), 1.38 (3H, d, J = 7.0 Hz), 1.10 (3H, s), 1.07 (3H, s). ESI-HRMS: m/z $[M+H]^+$ 1313.42439 (found), 1313.43419 (calcd).
26. *Spectral data for* ***3b***. ^{1}H NMR (500 MHz, CD_3OD): δ 8.45 (1H, d, J = 1.5 Hz), 8.00 (1H, dd, J = 2.0, 8.0 Hz), 7.80 (2H, m), 7.63 (1H, m), 7.58 (1H, m), 7.48–7.16 (11H, m), 6.66 (2H, dd, J = 3.5, 6.5 Hz), 6.58 (2H, d, J = 7.5 Hz), 6.31 (1H, s), 6.07 (1H, t, J = 8.5 Hz), 5.61 (1H, dd, J = 7.5, 10.5 Hz), 5.59 (1H, d, J = 7.0 Hz), 5.55 (1H, d, J = 5.5 Hz), 4.96 (1H, d, J = 9.0 Hz), 4.72 (1H, d, J = 6.0 Hz), 4.61 (1H, q, J = 7.0 Hz), 4.20 (1H, d, J = 8.0 Hz), 4.14 (1H, d, J = 8.0 Hz), 3.87 (1H, d, J = 7.0 Hz), 3.84 (3H, s), 2.57 (1H, ddd, J = 5.5, 8.0, 15.0 Hz), 2.29 (3H, s), 2.15 (1H, dd, J = 9.5, 15.5 Hz), 2.12 (3H, s), 1.90 (1H, dd, J = 9.5, 15.5 Hz), 1.87 (3H, s), 1.79 (1H, overlapped), 1.77 (3H, s), 1.39 (3H, d, J = 7.5 Hz), 1.10 (3H, s), 1.07 (3H, s). ESI-HRMS: m/z $[M+H]^+$ 1349.40696 (found), 1349.41535 (calcd).
27. *Spectral data for* ***4***. ^{1}H NMR (500 MHz, CD_3OD): δ 8.44 (1H, s), 8.08 (1H, dd, J = 2.0, 8.0 Hz), 7.84 (1H, d, J = 7.5 Hz), 7.73 (1H, m), 7.52 (1H, t, J = 8.0 Hz), 7.37–7.19 (7H, m), 6.79 (2H, d, J = 9.0 Hz), 6.62 (2H, d, J = 2.0 Hz), 6.53 (2H, dd, J = 2.0, 9.0 Hz), 6.38 (1H, q, J = 7.0 Hz), 6.32 (1H, s), 6.06 (1H, t, J = 9.0 Hz), 5.62 (1H, d, J = 7.0 Hz), 5.61 (1H, dd, J = 6.5, 11.0 Hz), 5.42 (1H, d, J = 4.5 Hz), 4.96 (1H, d, J = 9.0 Hz), 4.61 (1H, d, J = 4.5 Hz), 4.58 (1H, q, J = 7.0 Hz), 4.19 (1H, d, J = 8.0 Hz), 4.13 (1H, d, J = 8.0 Hz), 3.88 (1H, d, J = 7.0 Hz), 2.56 (1H, ddd, J = 5.5 7.5, 14.5 Hz), 2.32 (3H, s), 2.21 (1H, dd, J = 9.5, 15.5 Hz), 2.13 (3H, s), 2.00 (1H, dd, J = 9.5, 15.5 Hz), 1.86 (3H, s), 1.79 (1H, overlapped), 1.78 (3H, s), 1.76 (3H, s), 1.71 (3H, d, J = 7.0 Hz), 1.38 (3H, d, J = 7.0 Hz), 1.12 (3H, s), 1.08 (3H, s). ESI-HRMS: m/z $[M+H]^+$ 1302.44347 (found), 1302.44068 (calcd).