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# Synthesis and Microtubule Binding of Fluorescent Paclitaxel Derivatives

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**Abstract**—The preparation of two new fluorescent derivatives of paclitaxel in which the fluorophore is bonded to paclitaxel at the C-10 position is reported. Both analogues, 10-deacetyl-10-(*m*-aminobenzoyl)paclitaxel (**1**, BTax) and 10-deacetyl-10-[7-(diethylamino) coumarin-3-carbonyl]paclitaxel (**2**, CTax) retain good activity as promoters of in vitro tubulin assembly. Microtubule binding enhances the emission intensity of both probes. 

The complex natural product paclitaxel (Taxol®) (**3**), first isolated from *Taxus brevifolia*,[1] is a member of a large family of taxane diterpenoids.[2] Paclitaxel is widely used to treat solid tumors, particularly those of the breast and ovaries. The drug exerts its pharmacological effects by interacting with cellular microtubules.[3] Microtubules are primarily composed of tubulin, a 100 kDal heterodimer that reversibly assembles to form the core of the microtubule. Proper spatial and temporal control of microtubule dynamics is essential for cellular homeostasis. Paclitaxel disrupts the dynamic processes of microtubules by binding to the polymer at a single site on β-tubulin.[4]

Fluorescence spectroscopy is a powerful and versatile tool for the study of biological systems and has been extensively applied to the study of paclitaxel–microtubule interactions. Small, environmentally sensitive probes based on the aminobenzoate fluorophore have been used to explore the local environment of the paclitaxel binding site.[5–8] This fluorophore, however, must be excited with ultraviolet light, which limits its usefulness. Probes that are exited by visible light are more desirable. They are best suited for visualization of microtubule-bound paclitaxel using fluorescence microscopy and can be easily detected in plate readers. These structures tend to be large (rhodamine, fluorescein, and BODIPY) and their fluorescence emission spectra are relatively insensitive to their environment. These fluorophores are most commonly attached to paclitaxel through the C-7 position,[9–13] the most synthetically accessible site on the 'northern hemisphere' of paclitaxel (C-6–C-12 positions). Structural changes in this portion of the molecule appear to have less impact on its anti-microtubule activity than modifications in the 'southern hemisphere'.[14]

We have been probing the nature of the paclitaxel–tubulin interaction through synthesis and in vitro biological investigation of fluorescent paclitaxel derivatives. In our previous studies, we prepared and investigated paclitaxels in which the fluorophore is contained in the 'southern hemisphere' (i.e., at C-2 and C-3′).[7,8] We chose the less utilized C-10 position to initiate our investigations into the 'northern hemisphere' of paclitaxel. Moreover, we chose to use 7-(diethylamino)coumarin-3-carboxylic acid as one of the labels. This fluorophore has not yet been used as a probe for the paclitaxel–tubulin interaction. Other coumarin derivatives have been used to label paclitaxel, but the resulting probes had absorption maxima at high energy (~370 nm).[10,13] Inclusion of an electron withdrawing group at the 3-position of 7-(diethylamino)coumarin yields a probe that is excited by visible light (410–430 nm) and is also environmentally sensitive.[15] Reported herein are the synthesis of two C-10 modified fluorescent paclitaxels and their in vitro tubulin binding properties.

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## Results

### Synthesis of fluorescent paclitaxels modified at the C-10 position

The sequence leading to the required fluorescent paclitaxels is shown in Scheme 1. The known paclitaxel derivative (**4**)[16] was esterified at the C-10 position with 3-nitrobenzoic acid, employing the carbodiimide-based coupling protocol to yield the paclitaxel analogue **5**. Concomitant removal of both silyl protecting groups, followed by hydrogenation, gave the desired amine (**1**, BTax) in good yield. CTax (**2**) was prepared from the common intermediate **4** by acylation at C-10 as before utilizing 7-(diethylamino)coumarin-3-carboxylic acid, followed by simultaneous removal of the protecting groups.[17]

### Microtubule activity

The activities of the ligands were assessed by their abilities to induce purified tubulin to assemble in vitro. Both Btax and CTax were nearly equipotent to paclitaxel in promoting tubulin assembly, although CTax did not promote assembly quite as rapidly as paclitaxel (Fig. 1).

The absorption, excitation and emission spectra of the two fluorescent ligands were obtained in a variety of solvents and bound to microtubules. The effect of microtubule binding on the emission spectra of the compounds is illustrated in Figure 2. The emission intensity of BTax increased and underwent a small blue shift upon microtubule binding (Fig. 2A). Since the absorption maximum of the ligand in buffer is near 320 nm, ultraviolet radiation is required for excitation of this fluorophore. CTax possesses photochemical properties that will make it more useful than BTax as a fluorescent probe. The absorption and emission maxima are both in the visible region of the electromagnetic spectrum. The emission intensity is very strongly affected by environmental polarity, while the emission energy is less so (Table 1). Microtubule binding by CTax is accompanied by a significant increase in emission intensity (Fig. 2B). The appearance of fluorescence coincides with assembly of the protein (Fig. 3), which further demonstrates that the fluorescence changes that occur in CTax in the presence of tubulin are due to microtubule binding.

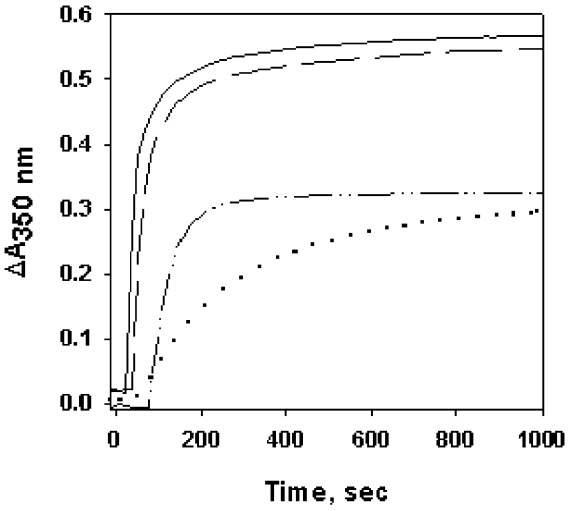
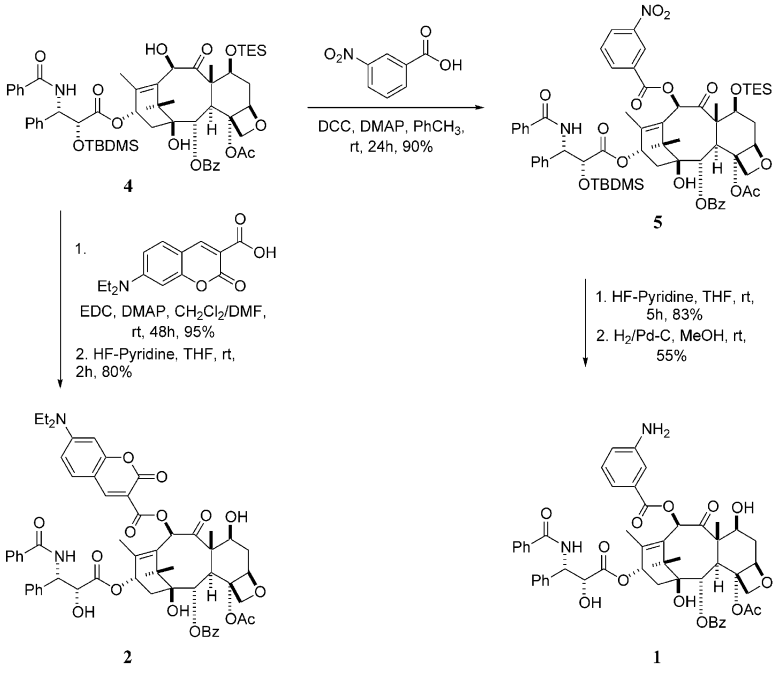



**Figure 1.** Promotion of tubulin assembly by paclitaxel, BTax and CTax. Tubulin in PMEG buffer (0.1 M Pipes, 1 mM $MgSO_4$, 2 mM EGTA and 0.1 mM GTP, pH 6.9) was equilibrated to 37 °C prior to addition of the ligand. Assembly was monitored by apparent light scattering (absorption at 350 nm). Solid curve: 10 μM BTax; dashed curve: 10 μM paclitaxel. Dash dot dot curve: 5 μM paclitaxel; dotted curve: 5 μM CTax.




**Scheme 1.** Synthesis of fluorescent paclitaxel derivatives.

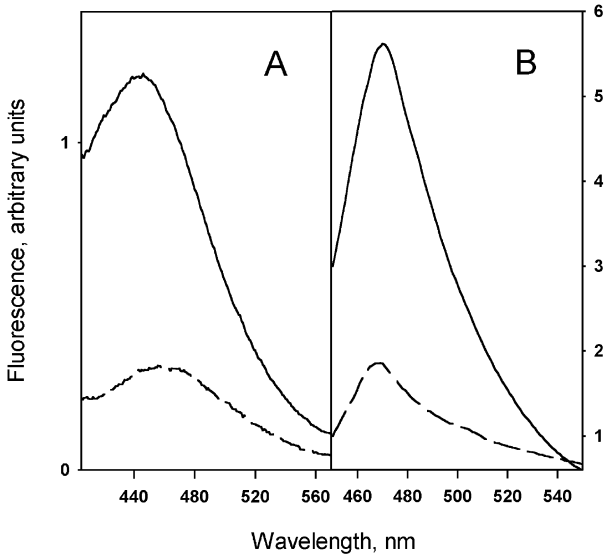



**Figure 2.** Emission spectra of BTax and CTax in the presence and absence of tubulin. (A) Solid curve: BTax (10 μM) in PMEG buffer was incubated with 10 μM tubulin at 37 °C prior to collection of the emission spectrum. Dashed curve: Emission spectrum of 10 μM BTax in PMEG buffer. The excitation wavelength was 320 nm. (B) Solid curve: CTax (0.4 μM) in PMEG buffer was incubated with 5 μM tubulin at 37 °C prior to collection of the emission spectrum. Dashed curve: Emission spectrum of 0.4 μM CTax in PMEG buffer. The excitation wavelength was 420 nm.

**Table 1.** Absorption and emission maxima and relative emission intensity of CTax in solvent and bound to microtubules

| Solvent | Absorption maximum (nm) | Emission maximum (nm) | Relative fluorescence intensity[a] |
|---|---|---|---|
| Dioxane | 413 | 447 | 42 |
| Ethyl acetate | 415 | 452 | 40 |
| Dimethylsulfoxide | 427 | 469 | 18 |
| Acetonitrile | 422 | 464 | 1.0 |
| Ethanol | 423 | 461 | 0.11 |
| Methanol | 424 | 462 | 0.27 |
| 2% DMSO/water | 428[b] | 462[b] | 0.06 |
| Microtubules | 430[c] | 468 | 4.8[d] |

[a]For solvents: intensity at emission maximum, normalized to acetonitrile value. The absorptivity of the solvent samples at the excitation wavelength (420 nm) was equal. For microtubules: intensity of bound drug relative to free drug.
[b]Without DMSO cosolvent and at higher concentrations the ligand undergoes self-association. The absorption maximum of aggregated samples is 434 nm and the emission maximum is 530 nm.
[c]Approximate value due to turbidity of microtubule-containing solution.
[d]Concentration of free drug at 0.4 μM, concentration of bound drug at 0.4 μM in presence of 5 μM tubulin.

The small change in emission energy observed when BTax and CTax bind to microtubules indicates that the substituent is solvent accessible. This observation is consistent with the structure–activity data for C-10 analogues of paclitaxel.[18] Based on their spectroscopic observations, Evangelio et al.[13] and Sengupta et al.[6] hypothesized that the C-7 and C-10 position of paclitaxel may be in a cationic microenvironment. These predictions were made before the electron crystallographic structure of tubulin was published.[19] It is now known that tubulin has at least two arginine residues located on the exterior of the paclitaxel binding site,[19] and at least one of these is in close proximity to the C-7 position of the tubulin-bound ligand.[20] The spectroscopic data for microtubule-bound Btax and CTax are also consistent with the presence of a charged amino acid in close proximity to the fluorophore.

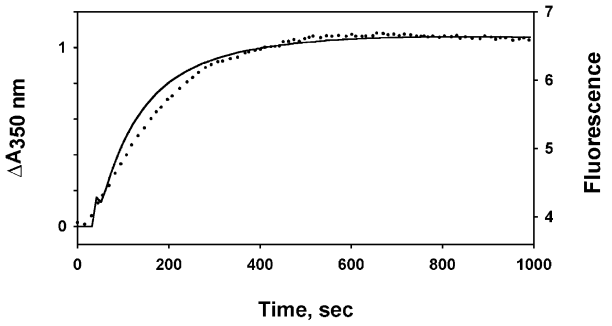



**Figure 3.** Promotion of tubulin assembly by CTax. Tubulin (10 μM) in PMEG buffer was equilibrated to 37 °C prior to addition of 10 μM CTax. Assembly was monitored by apparent light scattering (dotted curve) or by emission at 465 nm (solid curve; excitation at 420 nm).

## Conclusion

The C-10 position can be used to prepare fluorescent derivatives of paclitaxel that retain good in vitro assembly-promoting activity. The small change in emission intensity when BTax binds to microtubules limits its utility as a probe. The 7-(diethylamino)coumarin-3-carbonyl fluorophore, which has not been previously used to fluorescently label paclitaxel, produces a probe with many possible utilities. Since the probe can be excited with visible light, the fluorescent paclitaxel-microtubule association can be readily observed in standard plate readers and possibly in live cells. Unlike the rhodamine and fluorescein paclitaxel derivatives,[13] the emission intensity of the diethylaminocoumarin fluorophore in CTax undergoes a large change in emission intensity in upon microtubule binding. Thus, background fluorescence due to unbound ligand will be low when CTax is used as a probe.

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16. Paclitaxel derivative **4** was prepared by protecting the known 10-deacetyl-2′-(*tert*-butyldimethylsilyl) paclitaxel as its triethylsilyl ether at C-7 position. Datta, A.; Hepperle, M. I. G. G. *J. Org. Chem.* **1995**, *60*, 761.
17. General procedure for esterification at the C-10 position: 1 equivalent of the carboxylic acid was stirred in toluene at room temperature with 1 equivalent of dicyclohexyl carbodiimide (DCC) or 1-[3-(dimethylamino)propyl-3-ethylcarbodiimide hydrochloride (EDC) for 15 min. 0.01 equivalent of 4-(dimethylamino)pyridine (DMAP) was added and stirred for 5 min. 0.1 equivalent of 10-deacetyl-2′-(*tert*-butyldimethylsilyl)-7-(triethylsilyl) paclitaxel was then introduced and stirred at rt for 24–48 h. In order to overcome the solubility problem of 7-(diethylamino)coumarin-3-carboxylic acid, $CH_2Cl_2$/DMF (10:1) solvent system was used for **2**. The progress of the reaction was monitored by thin layer chromatography and when the reaction was complete, the reaction mixture was diluted with EtOAc. The organic layer washed with water (×2), $NaHCO_3$ (×2), brine and dried over sodium sulfate. The crude product was applied on a preparative thin layer chromatography plate, developed with solvent system of 40% EtOAc/hexane to give the desired products. $^1$H NMR of **1** (400 MHz, $CDCl_3$) δ 8.12–8.14 (d, 2H), 7.72–7.48 (d, 2H), 7.61 (t, 1H), 7.34–7.53 (m, 12H), 7.24 (t, 1H), 7.02–7.05 (d, 1H), 6.88–6.90 (dd, 1H), 6.49 (s, 1H), 6.24 (t, 1H), 5.77–5.80 (dd, 1H), 5.70–5.72 (d, 1H), 4.95–4.97 (d, 1H), 4.80 (s, 1H), 4.47 (m, 1H), 4.30–4.32 (d, 1H), 4.19–4.21 (d, 1H), 3.85–3.87 (d, 1H), 3.82 (bs, 2H), 3.62 (bs, 1H), 2.66–2.67 (d, 1H), 2.57 (m, 1H), 2.39 (s, 3H), 2.34 (m, 1H), 1.89 (m, 1H), 1.81 (s, 3H), 1.69 (s, 3H), 1.66 (s, 3H), 1.30 (s, 3H), 1.24 (s, 3H); $^{13}$C NMR of **1** (100 MHz) δ 203.45, 172.66, 170.38, 167.05, 166.97, 166.57, 146.56, 142.07, 137.89, 133.72, 133.59, 133.24, 131.96, 130.19, 129.97, 129.41, 129.13, 129.01, 128.72, 128.69, 128.37, 127.02, 120.17, 120.08, 115.99, 84.44, 81.18, 79.04, 76.49, 75.85, 74.95, 73.17, 72.38, 72.25, 58.66, 55.03, 45.72, 43.22, 35.72, 35.68, 29.68, 27.04, 22.63, 22.07, 14.88, 9.57. HR-FABMS of **1** *m*/*z* found 953.343872 $(M+Na)^+$, calcd 953.3473 $(M+Na)^+$; LR-FABMS *m*/*z* found 953.5 $(M+Na)^+$. $^1$H NMR of **2** (400 MHz, $CDCl_3$) δ 8.48 (s, 1H), 8.12–8.14 (d, 2H), 7.74–7.76 (d, 2H), 7.61 (t, 1H), 7.34–7.53 (m, 11H), 7.05–7.07 (d, 1H), 6.60–6.63 (dd, 1H), 6.52 (s, 1H), 6.45–6.46 (d, 1H), 6.25 (t, 1H), 5.78–5.80 (dd, 1H), 5.70–5.72 (d, 1H), 4.95–4.97 (d, 1H), 4.801–4.807 (d, 1H), 4.40–4.48 (m, 1H), 4.30–4.32 (d, 1H), 4.20–4.22 (d, 1H), 3.86–3.88 (d, 1H), 3.43–3.49 (q, 4H), 2.57 (m, 1H), 2.40 (s, 3H), 2.30–2.34 (m, 3H), 1.87–1.94 (m, 2H), 1.82 (s, 3H), 1.71 (s, 3H), 1.32 (s, 3H), 1.23–1.28 (m, 15H); $^{13}$C NMR of **2** (100 MHz) δ 203.65, 172.56, 170.40, 166.98, 164.32, 158.80, 153.23, 150.22, 141.79, 137.89, 133.68, 133.63, 133.18, 131.92, 131.47, 130.19, 129.16, 128.99, 128.68, 128.34, 127.04, 109.73, 107.84, 107.37, 69.77, 84.44, 81.18, 79.04, 76.49, 75.73, 74.96, 73.20, 72.38, 72.19, 58.69, 55.01, 45.79, 45.19, 43.27, 35.74, 35.66, 26.57, 22.63, 21.66, 14.92, 12.43, 9.58 LR-FABMS $(M+H)^+$ *m*/*z* found 1055.6.
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