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Synthesis and Biological Evaluation of a Biotinylated Paclitaxel with an Extra-Long Chain Spacer Arm

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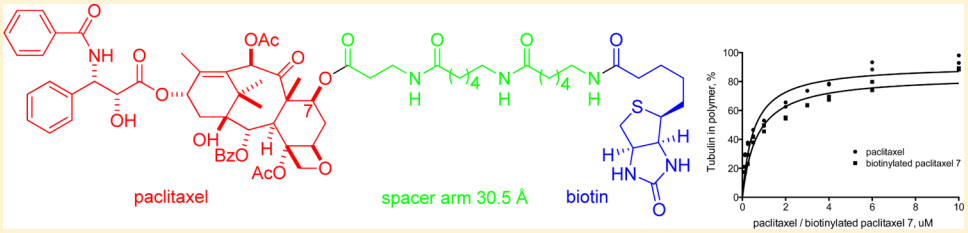
Supporting Information



ABSTRACT: A biotinylated paclitaxel derivative with an extra-long chain (LC-LC-biotin) spacer arm was synthesized using an improved synthetic reaction sequence. The biotinylated paclitaxel analogue retained excellent microtubule stabilizing activity in vitro. Furthermore, it was shown that this analogue can simultaneously engage streptavidin and the binding site on microtubules, making it suitable for localization studies or for the attachment of paclitaxel to solid substrates via a streptavidin linkage.

KEYWORDS: *paclitaxel, taxol, biotin, biotinylated*

Paclitaxel **1** (Taxol, Scheme 1)[1,2] is a complex diterpene natural product that was first isolated in 1971 from the stem bark of the western yew, *Taxus brevifolia*.[3] It is widely used as an anticancer chemotherapeutic drug to treat a variety of solid tumors such as breast, ovarian, nonsmall cell lung, and head and neck cancers.[2,4−12] Biotin (vitamin B_7, vitamin H) is an essential cellular micronutrient responsible for various normal cellular functions. Various cancer cell lines are known to overexpress biotin receptors,[13] and it has been suggested that the sodium-dependent multivitamin transporter (SMVT) is the primary transporter responsible for biotin uptake.[14] SMVT expression in several lung, renal, colon, and breast cancer cell lines is higher than that of the folic acid receptor.[15] Therefore, it is possible that a biotin conjugate may be more efficient for targeting tumors than using a folic acid conjugate.[15] This overexpression makes it possible to use biotinylation as a strategy for selective delivery of biotinylated anticancer agents to cancer cells.[15−19]

Paclitaxel **1** represents a class of clinically proven anticancer agents that function by promoting microtubule assembly and suppressing microtubule dynamics, leading to mitotic arrest and apoptosis.[20] Understanding how compounds like paclitaxel exert influence over microtubule dynamics holds potential for optimizing microtubule-based therapies. Under saturating conditions, paclitaxel binds to microtubules at a 1:1 molar ratio to tubulin.[21] However, the dynamics of microtubules are sharply suppressed when paclitaxel-like compounds are bound at very low stoichiometry.[22,23] Currently, the binding arrangement of paclitaxel under these conditions, that is, spread throughout the lattice or bound preferentially to microtubule ends, is not known. One confounding factor has been that although the paclitaxel binding site on tubulin is oriented toward the interior lumen of the microtubule, paclitaxel can diffuse through the 1−2 nm fenestrations present in the microtubule lattice. Thus, paclitaxel can readily exchange into preformed or existing polymer.[24] Paclitaxel−biotin conjugates that were previously reported by Rosen[25] and Hwu[26] contained shorter linkers (16 and 4 atoms, respectively). To prevent access through fenestrations or other similar access points that may exist along the lattice, we sought to attach paclitaxel to a relatively large molecule such as streptavidin. To facilitate the ability of the paclitaxel−biotin conjugate to simultaneously engage streptavidin and the binding site on tubulin within the microtubule, we sought to maximize the intervening linker arm. To achieve that goal, we took advantage of a commercially available biotin with an extra-long chain spacer.

Our synthetic strategy was based on the approach earlier reported by Nicolaou and co-workers[27] for fluorescent taxoid synthesis and later expanded by Rosen and co-workers[25] for biotin−taxol conjugation using *N*-hydroxysuccinimide (NHS) esters of biotin as convenient biotinylation reagents. A similar approach was reported by Lee and co-workers for paclitaxel−-camptothecin conjugates.[28] NHS is a good leaving group, and NHS-activated biotins react easily with primary amino groups forming stable amide bonds. The synthetic sequence is shown

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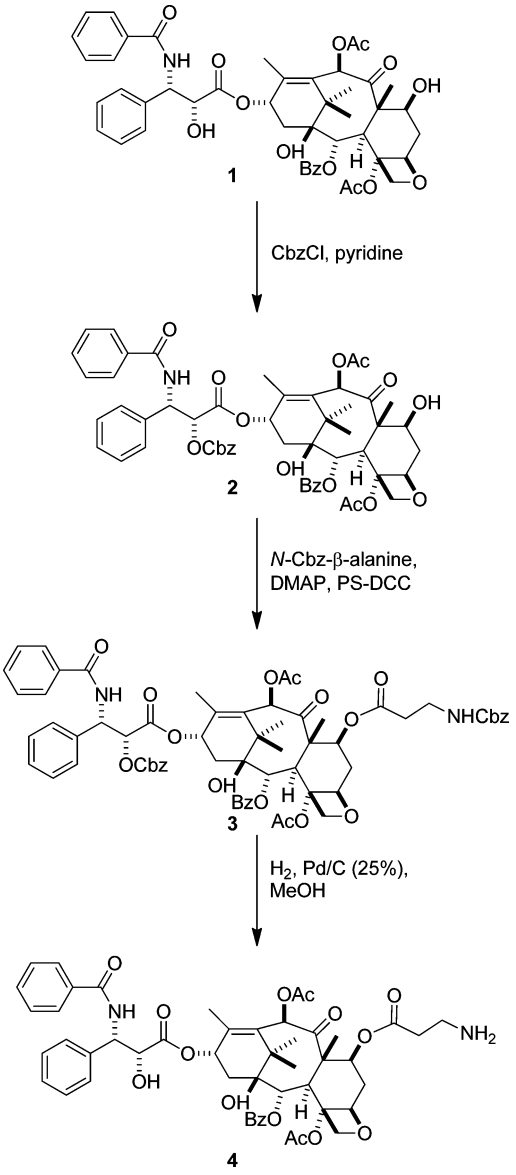
Scheme 1. Synthesis of 7-(β-Alanyl)paclitaxel 4



in Scheme 1. In the first step, the 2′-hydroxy group of paclitaxel **1** was protected by carboxybenzylation. The resultant 2′-carboxybenzyl derivative **2** was subjected to esterification at the 7-hydroxy group. The 2′-hydroxy group is protected, and the 1-hydroxy group is unreactive; therefore, the subsequent acylation takes place selectively at the 7-hydroxy group. It was previously reported that α-amino esters at the 7-hydroxy group of paclitaxel are very unstable.[29] As an alternative, β-, γ-, or ω-amino acid esters, which are far more stable, can be used.[27,30,31] We employed *N*-carboxybenzoyl-β-alanine[32] for esterification of the 7-hydroxy group, resulting in compound **3**. The next step, deprotection of both carboxybenzyl groups by hydrogenation, led to the formation of key intermediate **4**.

Our study revealed that deprotection of compound **3** by hydrogenation is very sensitive to the reaction time, amount of solvent, and amount of catalyst. A significant formation of degradation products was observed when the reaction time exceeded 2.5 h. Using a more diluted solution (4.2−6.7 mM) of substrate **3** (as compared to the previously reported 21 mM)[27] increased the reaction time from 2 to 18 h and the level of degradation. At the previously reported substrate−catalyst ratio (20:1 by weight),[27] deprotection is likely to occur on the 2′-position initially, producing monodeprotected compound **5** (Figure 1).[33] Its structure was confirmed by nuclear magnetic resonance (NMR) analysis (for NMR data for compounds **4** and **7** and main byproduct **5**; see the Supporting Information).

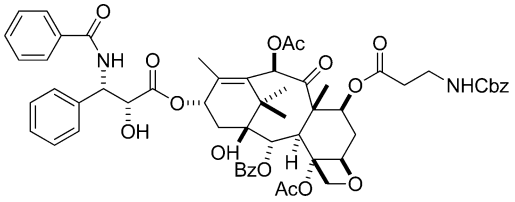
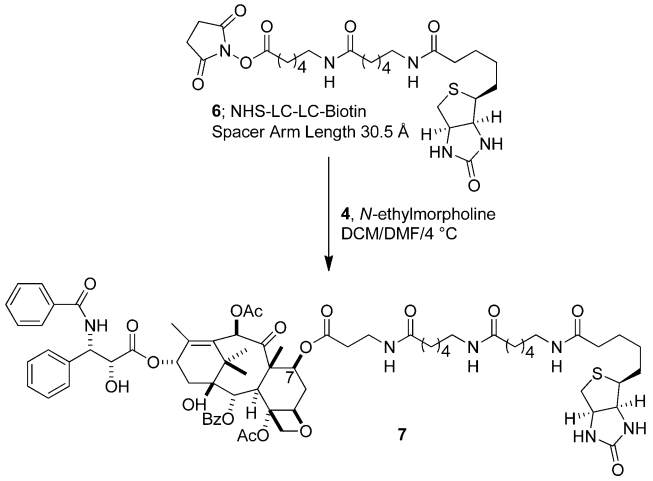


Figure 1. Partially deprotected compound **5**.

Compound **5** is far less soluble than substrate **3** and the product **4** and precipitated during the reaction. As a result, the process significantly slowed down, increasing the level of degradation of the substrate or product. Therefore, accelerating the hydrogenation rate is critical for this reaction. To achieve that goal, we increased the amount of the Pd−C catalyst 5-fold and used shaking instead of stirring to increase the efficiency of mixing. A slow stream of hydrogen gas in the reaction vessel allowed rapid removal of the carbon dioxide formed from the reaction. This protocol achieved a 90% yield of compound **4** within a 2 h period.

In the next step, compound **4** was reacted with the hydroxysuccinimide ester of LC-LC-biotin **6** (NHS-LC-LC-biotin) (Scheme 2). The reaction was carried out in in

Scheme 2. Biotinylation of 7-(β-Alanyl)paclitaxel 4



anhydrous mixture of dichloromethane (DCM) and dimethylformamide (DMF) in the presence of *N*-ethylmorpholine at 4 °C for 24 h to produce paclitaxel-LC-LC-biotin **7**. Because the thin-layer chromatography (TLC) mobility of the product **7** is very similar to that of NHS-LC-LC-Biotin **6**, preparative HPLC was employed for the final purification of the product.

The ^{1}H NMR spectrum shows broad multiplets at 3.08−3.32 and 3.6 ppm (total seven protons), which are characteristic for the three $CH_2NC(=O)$ groups and the H_β of the CH_2S group of biotin. The signals at 4.25 and 4.45 ppm represent the two biotin CHN protons. The group of signals at 1.14−1.69 ppm is associated with the aliphatic protons of the spacer arm.

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We determined the relative activity of compound **7** in a tubulin assembly assay (Figure 2). The EC_{50} for stimulation of

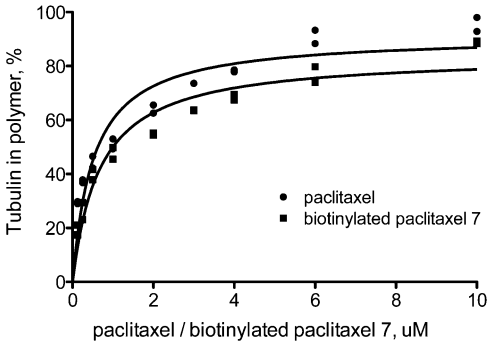

Figure 2. Tubulin assembly assay for paclitaxel (circles) and biotinylated paclitaxel **7** (squares). The EC_{50} was 0.52 ± 0.1 μM for paclitaxel **1** and 0.68 ± 0.1 μM for biotinylated paclitaxel **7**. The data points are from two independent experiments.

microtubule polymerization in vitro was 0.52 ± 0.1 and 0.68 ± 0.1 μM for paclitaxel **1** and the biotinylated paclitaxel **7**, respectively. Thus, the presence of the long linker biotin moiety at position 7 did not significantly impact the microtubule polymerizing and stabilizing activity of paclitaxel.

To determine whether biotinylated paclitaxel **7** could simultaneously bind to streptavidin and microtubules, we used biotinylated paclitaxel **7** prebound to fluorescent streptavidin to drive microtubule assembly. The microtubules were sedimented through a glycerol cushion to remove unbound streptavidin and paclitaxel before being visualized by fluorescent microscopy (Figure 3). Oregon green-labeled tubulin demonstrated that both paclitaxel and biotinylated paclitaxel **7** stabilized microtubules during this procedure.

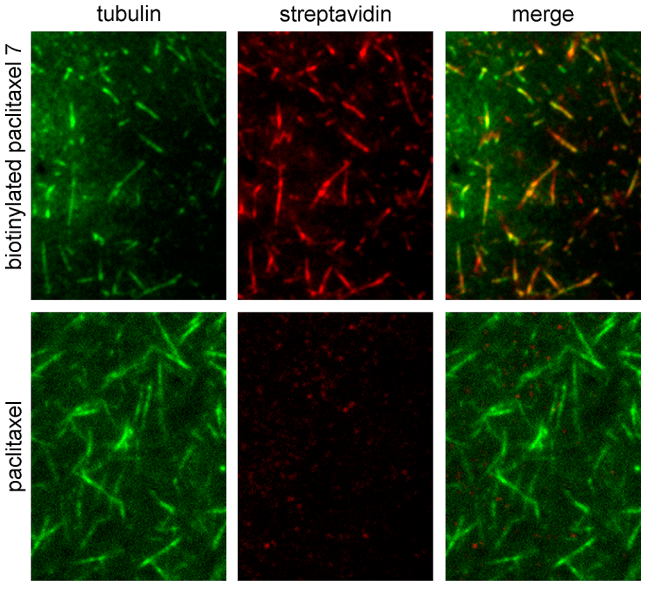

Figure 3. Biotinylated paclitaxel **7** can simultaneously engage microtubules and streptavidin. Microtubules were polymerized from Oregon green-labeled tubulin in the presence of Cy3-labeled streptavidin and either paclitaxel **1** (bottom row) or biotinylated paclitaxel **7** (top row). Microtubules were sedimented through a 40% glycerol cushion before being visualized by fluorescence microscopy for microtubules (left) and streptavidin (center).

Visualization of Cy3-labeled streptavidin confirmed that biotinylated paclitaxel **7** bound to streptavidin could also bind to tubulin during microtubule polymerization and be retained during sedimentation through the glycerol cushion and dilution prior to visualization. Microtubules assembled with paclitaxel in the presence of identical concentrations of fluorescent streptavidin did not contain any Cy3 signal associated with the polymer, demonstrating that streptavidin was not simply constrained within the microtubules during polymerization but was bound to biotinylated paclitaxel **7** during polymerization and isolation of the microtubules.

In summary, we developed an improved process for the synthesis of the key intermediate amine **4** that is commonly used[25,26,27,28,30,31,34,35] in biotinylation of taxol derivatives. Using this process, we synthesized a biotinylated paclitaxel derivative with the longest spacer arm reported as of yet. This long spacer arm did not significantly disrupt the microtubule stabilizing activity of paclitaxel, yet allowed microtubule-bound paclitaxel to engage streptavidin through the biotin moiety. This compound, bound to streptavidin, can be utilized in biochemical studies to both stabilize microtubules and restrict paclitaxel access through microtubule fenestrations. Biotinylated paclitaxel **7** could also potentially be used to attach paclitaxel to solid supports or for selective delivery into cells.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed experimental priocedures, copies of ^{1}H NMR and ^{13}C NMR, MS, and UPLC chromatogram for the final product **7**, and analytical data for key intermediate **4** and main byproduct **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Cy3, 3*H*-indolium, 2-[3-[1-[6-[(2,5-dioxo-1-pyrrolidinyl)oxy]-6-oxohexyl]-1,3-dihydro-3,3-dimethyl-5-sulfo-2*H*-indol-2-ylidene]-1-propen-1-yl]-1-ethyl-3,3-dimethyl-5-sulfo-, inner salt; DCM, dichloromethane; DMF, dimethylformamide; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography

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