Synthesis of a Photoaffinity Analog of Taxol as an Approach To Identify the Taxol Binding Site on Microtubules

Joan M. Carboni,* Vittorio Farina,† Srinivasa Rao,‡ Sheila I. Hauck,† Susan Band Horwitz,‡ and Israel Ringel§

Department of Experimental Therapeutics, and Antitumor Chemistry, Bristol Myers Squibb Research Institute, Wallingford, Connecticut 06492, Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461, and Department of Pharmacology, The Hebrew University-Hadassah Medical School, Jerusalem, 91010, Israel

Received September 8, 1992

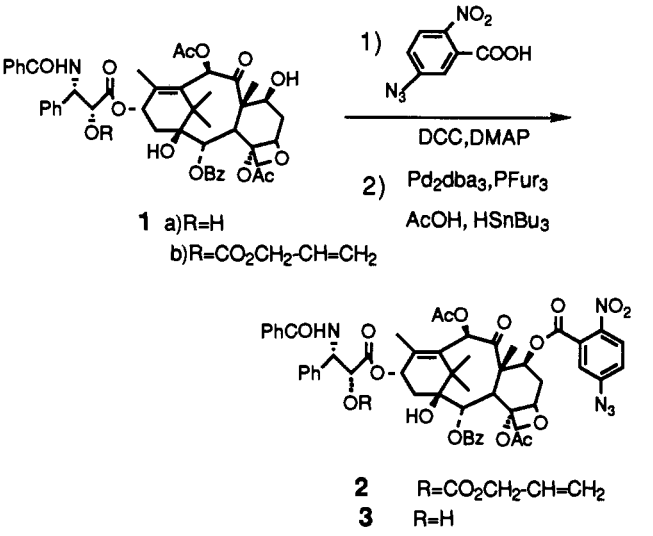
Taxol, a plant product extracted from the bark of the Western yew, *Taxus brevifolia*,[1] is a potent inhibitor of cell replication that blocks cells in the mitotic phase of the cell cycle.[2] Unlike colchicine and the vinca alkaloids, which prevent microtubule assembly, taxol promotes the polymerization of stable microtubules.[3] This action of taxol renders microtubules resistant to depolymerization and inhibits their normal functions.

The molecular mechanism(s) through which taxol exerts its effects, both in vivo and in vitro, has been the subject of extensive research during the past decade; however, little is known regarding the binding site for taxol on the microtubule. To this end, a photoaffinity (PA) analog of taxol was synthesized and is currently being investigated for its ability to covalently bind to microtubules. Elucidation of the taxol-microtubule binding domain is of importance in the design of new drugs and analogs with taxol-like activity.

Chemistry. Synthesis of PA Analog. Tubulin is sensitive to irradiation at 254 nm.[4] Therefore, we selected a photoaffinity probe with a UV maximum at a higher wavelength. The 5-azido-2-nitrobenzoic acid probe, with a maximum absorption at approximately 310 nm,[5] may be a suitable candidate for tubulin photoaffinity binding. Since the 2′-hydroxyl group of taxol is involved in binding, we utilized the 7-hydroxyl group, which can be esterified without loss of activity.[6] The synthesis of the photoaffinity analog compound **3** is depicted in Scheme I

Taxol, **1a**, was first protected at the reactive C-2′ hydroxyl group. Due to the sensitivity of the azide function being introduced, we chose the allyloxycarbonyl (alloc) group, which can be readily removed with Pd(0) and tributyltin hydride.[7] The C-7 hydroxyl group was then acylated with 5-azido-2-nitrobenzoic acid and dicyclohexylcarbodiimide (DCC). Final removal of the alloc group was troublesome. Under the typical literature conditions,[7] we found that triphenylphosphine was consumed by the substrate to produce a bright yellow product, which inactivated the catalyst. Presumably the phosphine is too nucleophilic and reacts competitively with the very electrophilic aryl azide to form a yellow phosphinimine.

Scheme I



When a much less nucleophilic phosphine was used, i.e. tri-2-furylphosphine,[8] no competing side reaction took place and the desired product was obtained in high yield (supplementary material is available).

Biological Activity. In Vitro Polymerization. Under physiological conditions, microtubule assembly requires GTP, whereas in the presence of taxol this requirement is eliminated. The kinetics of microtubule assembly was determined in the presence of the PA analog (Figure 1). The PA analog was capable of enhancing the assembly of microtubules in vitro. The initial slopes for taxol and the PA analog, in the absence of GTP, were 0.033 and 0.008 AU/min, respectively. Furthermore, polymers induced by both taxol and the PA analog were stable to depolymerization by calcium whereas those assembled in the presence of GTP were not. Electron microscopic studies further revealed that the polymers formed by the PA analog were similar to those obtained in the presence of taxol (data not shown).

Competition Assay. The ability of the PA analog to inhibit the binding of [^{3}H]taxol was determined as previously described.[9,10] The PA analog competed with [^{3}H]taxol for its binding site on the polymer with an apparent K_i of 10 μM compared to 2 μM for taxol (Figure 2). Similar studies that corroborated these results were done by HPLC as previously described.[10] These data suggest that the PA analog and taxol bind to a common or overlapping region on the microtubule, but that the PA analog binds with a lower affinity compared to taxol.

Cytotoxicity. The cytotoxicity of the PA analog was compared to that of taxol. This assay gives information that correlates well with tubulin polymerization and binding data.[11] The ED_{50} (drug concentration that inhibits cell division by 50% after 72 h of incubation) of the PA analog for the murine-like macrophage cell line, J774.2, was 0.16 μM compared to 0.05 μM for taxol.

Immunofluorescence. To determine whether the PA analog, like taxol, induces the formation of stable microtubule bundles in cultured cells, the immunofluorescent localization of tubulin was assessed (Figure 3). The PA analog induced microtubule bundle formation and mimicked the effect of taxol in the cell[2] (Figure 3). To be certain that the PA analog, and not a metabolite or

* To whom requests for reprints should be addressed, at Department of Experimental Therapeutics, P.O. Box 4000, Princeton, NJ 08543
† Antitumor Chemistry, Bristol Myers Squibb Research Institute.
‡ Department of Molecular Pharmacology, Albert Einstein College of Medicine.
§ Department of Pharmacology, The Hebrew University-Hadassah Medical School.

0022-2623/93/1836-0513$04.00/0

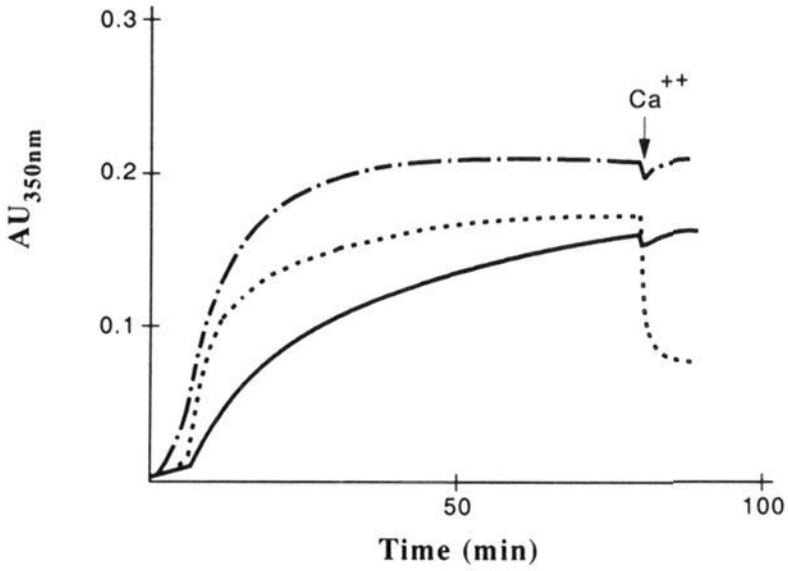


Figure 1. Polymerization of microtubules in vitro: Calf brain tubulin was prepared by two cycles of assembly–disassembly (2X MTP) according to the method of Shelanski et al.[18] Taxol was obtained from the National Cancer Institute, dissolved in 100% dimethyl sulfoxide, and stored frozen at −20 °C. The maximum final concentration of dimethyl sulfoxide was 1%. This concentration has no significant effect on tubulin polymerization. 2X MTP protein was incubated with 10 μM taxol (– · –) or 10 μM PA analog (—) in the absence of GTP, or with 1 mM GTP alone (- -). The assembly of microtubules was measured at 37 °C by a change in optical density at 350 nm. At the time indicated, $CaCl_2$ (Ca^{2+}) was added at a final concentration of 4 mM.

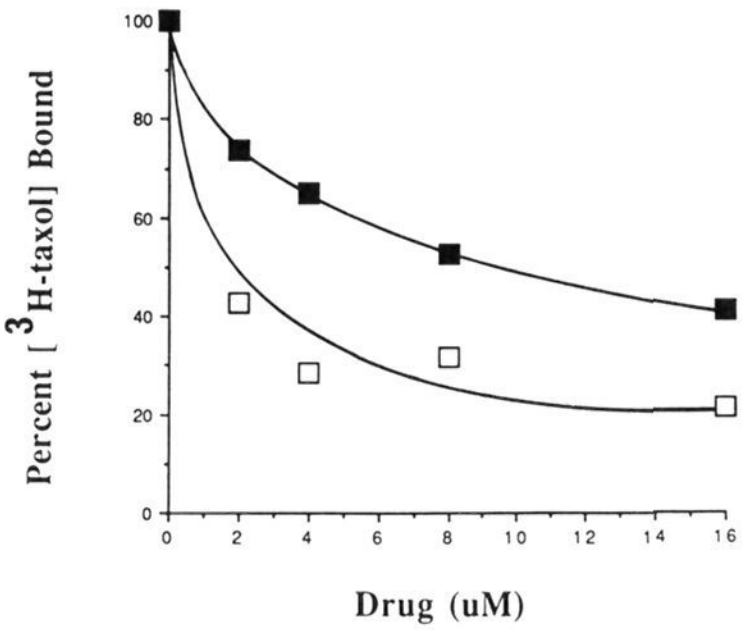


Figure 2. Competition Assay: a competition assay was performed using 2X MTP in the presence of 2 μM [^{3}H]taxol (0.34 μg, specific activity = 2.64 μCi/μg) and 1 mM GTP, with increasing amounts of unlabeled taxol or PA analog. [^{3}H]Taxol was prepared by an exchange procedure, using tritiated water, developed by Amersham International, Arlington Heights, IL. After incubation at 37 °C for 60 min, the reaction mixture was centrifuged in an airfuge for 30 min at 28 psi. The pellets were washed three times in reaction buffer (0.1 M MES, 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM GTP, pH 6.6) with a 5-min centrifugation at 28 psi between each wash, resuspended in 0.1 N NaOH, and analyzed for radioactivity and protein content using the BCA protein assay (Pierce Biochemicals, Rockford, IL).

breakdown product such as taxol, was responsible for the formation of stable microtubule bundles, cells were incubated with 10 μM taxol for 8 h, lysed, and extracted with methylene chloride as previously described.[10] HPLC analysis confirmed that the PA analog remained stable for the duration of the experiment and that taxol was not present.

Discussion. In this study, the synthesis and characterization of a C-7 substituted photoreactive taxol analog have been described. This analog was synthesized in order to explore the specific taxol binding site(s) on the

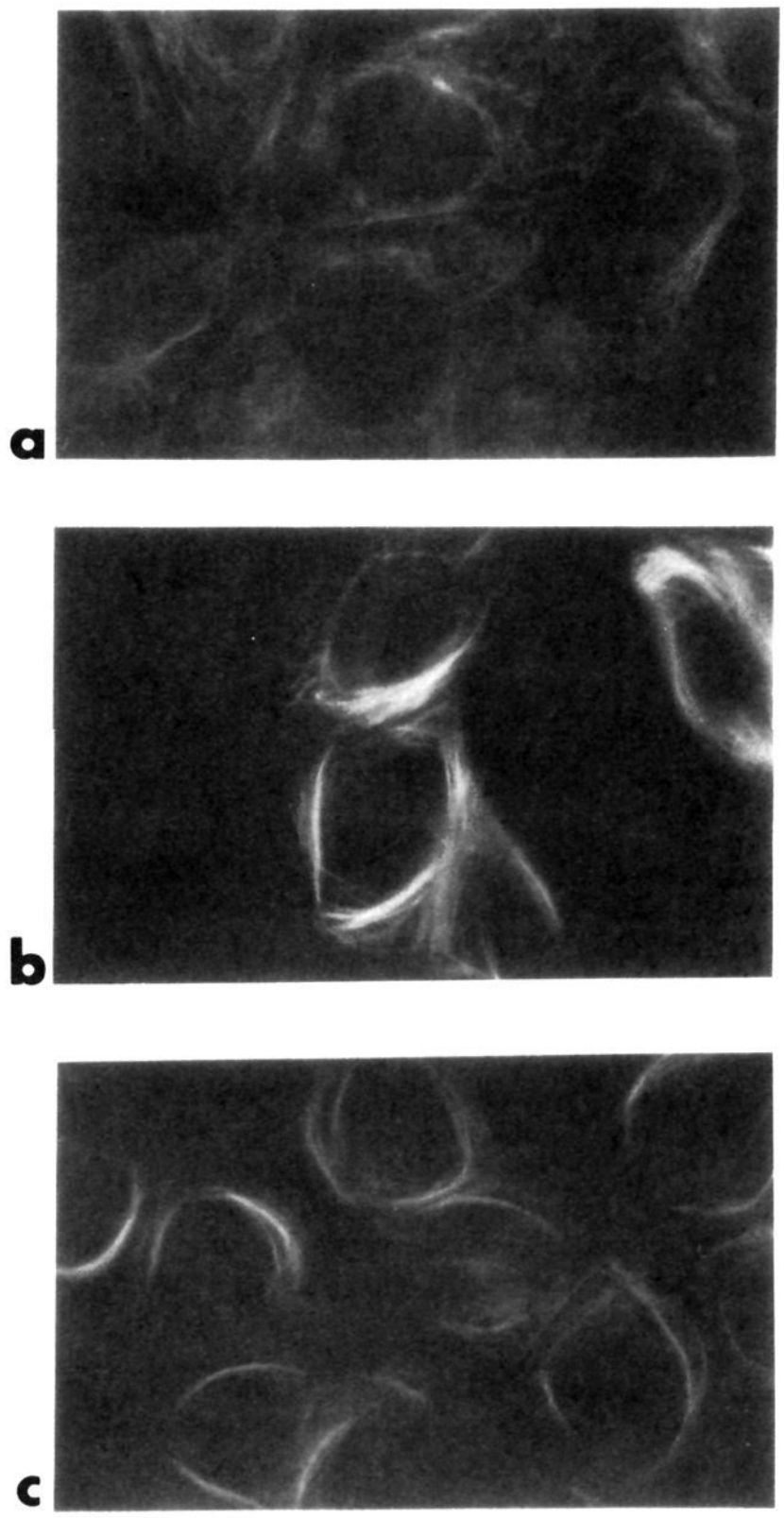


Figure 3. Indirect Immunofluorescence: HCT 116 colon adenocarcinoma cells (1.5×10^6/mL) were grown on coverslips coated with 0.01% poly(L-lysine). Cells were incubated with (a) DMSO (0.1%), (b) taxol, or (c) PA analog (10 μM) for 3 h at 37 °C. The coverslips were fixed in 3.7% formaldehyde, permeabilized in −20 °C acetone (2 min), and processed for immunofluorescence using a primary monoclonal antibody to β-tubulin, followed by fluorescein–goat F(ab′)$_2$ anti-mouse IgG.

microtubule. The PA analog retained taxol-like activity on the basis of several criteria: the ability to induce the assembly of microtubules in vitro and to compete with [^{3}H]taxol for its binding site on the polymer; cytotoxicity against J744.2 cells and induction of microtubule bundling in cells. In addition the PA analog was synthesized in relatively few steps, and exhibited a maximum excitation wavelength at 310 nm. The longer wavelength of excitation of this compound compared to the azido analogs lacking the nitro group is important to minimize photochemical damage to tubulin. Other photoreactive probes have been described which also bind to tubulin through an azido linkage, i.e., analogs of colchicine,[12] vinblastine,[13] and 7-(*p*-azidobenzoyl)taxol.[14] Recently, Kingston et al.[15,16] described the synthesis of a photoreactive analog of taxol that incorporated a C-ring 7-(azibenzoyl) group, but complete details have not been published. Synthesis of

a photoreactive side chain has also been described.[17] We are currently pursuing the synthesis of radiolabeled **3** and exploring the preparation of other photoaffinity probes.

Acknowledgment. We are grateful to Stephen G. Noell for excellent technical assistance.

Supplementary Material Available: Experimental details for the synthesis of **1b**, **2**, and **3** (3 pages). Ordering information is given on any current masthead page.

References

(1) Wani, M. C.; Taylor, H. L.; Wall, M. E.; Coggon, P.; McPhail, A. T. Plant Antitumor Agents. VI. The Isolation and Structure of Taxol, a Novel Antileukemic and Antitumor Agent from *Taxus brevifolia*. *J. Am. Chem. Soc.* **1971**, *93*, 2325–2327.

(2) Schiff, P. B.; Horwitz, S. B. Taxol Stabilizes Microtubules in Mouse Fibroblast Cells. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 1561–1565.

(3) Schiff, P. B.; Fant, J.; Horwitz, S. B. Promotion of Microtubule Assembly *In Vitro* by Taxol. *Nature* **1979**, *227*, 665–667.

(4) Zaremba, T. G.; LeBon, T. R.; Millar, D. B.; Smejkal, R. M.; Hawley, R. J. Effects of Ultraviolet Light on the *In Vitro* Assembly of Microtubules. *Biochemistry* **1984**, *23*, 1073–1080.

(5) Knowles, J. R. Photogenerated Reagents for Biological Receptor-site Labeling. *Acc. Chem. Res.* **1972**, *5*, 155–160.

(6) Mellado, W.; Magri, N. F.; Kingston, D. G. I.; Garcia-Arenas, R.; Orr, G. A.; Horwitz, S. B. Preparation and Biological Activity of Taxol Acetates. *Biochem. Biophys. Res. Commun.* **1984**, *124*, 329–336.

(7) Dangles, O.; Guibé, F.; Balavoine, G.; Lavielle, S.; Marquet, A.; Selective Cleavage of the Allyl and Allyloxycarbonyl Groups through Palladium-catalyzed Hydrostannolysis with Tributyltin Hydride. Application to the Selective Protection-deprotection of Amino Acid Derivatives and in Peptide Synthesis. *J. Org. Chem.* **1987**, *52*, 4984–4993.

(8) Farina, V.; Krishnan, B. Large Rate Accelerations in the Stille Reaction with Tri-2-furylphosphine and Triphenylarsine as Palladium Ligands: Mechanistic and Synthetic Implications. *J. Am. Chem. Soc.* **1991**, *113*, 9585–9595.

(9) Parness, J.; Horwitz, S. B. Taxol Binds to Polymerized Tubulin *In Vitro*. *J. Cell Biol.* **1981**, *91*, 479–487.

(10) Ringel, I.; Horwitz, S. B. Taxol is Converted to 7-Epitaxol, a Biologically Active Isomer in Cell Culture Medium. *J. Pharmacol. Exp. Ther.* *242*, **1987**, 692–698.

(11) Parness, J.; Kingston, D. G. I.; Powell, R. G.; Harracksingh, D.; Horwitz, S. B. Structure-activity Study of Cytotoxicity and Microtubule Assembly *In Vitro* by Taxol and Related Taxanes. *Biochem. Biophys. Res. Commun.* **1982**, *105*, 1082–1089.

(12) Williams, R. F.; Mumford, C. L.; Williams, G. A.; Floyd, L. J.; Aivaliotis, M. J.; Martinez, R. A., Robinson, A. K., Barnes, L. D. A Photoaffinity Derivative of Colchicine: 6′-(4′-Azido-2′-Nitrophenylamino) Hexanoyldeacetylcolchicine. *J. Biol. Chem.* **1985**, *260*, 13794–13802.

(13) Safa, A. R.; Hamel, E.; Felsted, R. L. Photoaffinity Labeling of Tubulin Subunits with a Photoactive Analogue of Vinblastine. *Biochemistry* **1987**, *26*, 97–102.

(14) Georg, G. I.; Harriman, G. C. B.; Himes, R. H.; Mejillano, M. R. Taxol Photoaffinity Label: 7-(p-Azidobenzoyl)taxol Synthesis and Biological Evaluation. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 735–738.

(15) Kingston, D. G. I.; Samaranayake, G.; Ivey, C. A. The Chemistry of Taxol, a Clinically Useful Anticancer Agent. *J. Nat. Prod.* **1990**, *53*, 1–12.

(16) Kingston, D. G. I. The Chemistry of Taxol. *Pharm. Ther.* **1991**, *52*, 1–34.

(17) Chatterjee, A.; Williamson, J. S.; Zjawiony, J. K.; Peterson, J. R. Synthesis of a Photoreactive Taxol Side Chain. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 91–94.

(18) Shelanski, M. L.; Gaskin, F.; Cantor, C. R. Microtubule Assembly in the Absence of Added Nucleotides. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 765–768.