An Improved Method for Separating Paclitaxel and Cephalomannine Using Ozone and Girard Reagents

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Received July 23, 1996

Paclitaxel (taxol A, **1**) is the active antitumor agent in the FDA-approved drug Taxol (a registered trademark of Bristol-Myers Squibb). Until recently, the only approved source of GMP bulk paclitaxel was via isolation from the bark of *Taxus brevifolia*.[1,2] A particularly difficult step in the purification of this compound from *Taxus brevifolia* bark or other *Taxus spp.* biomass is the separation from cephalomannine **2** (taxol B).[3−5] Cephalomannine differs from paclitaxel only in the amide portion of the molecule. It has been found that the tigloyl group of cephalomannine is susceptible to a variety of oxidizing conditions. Some of the oxidation methods are very selective for cephalomannine in a mixture of paclitaxel and cephalomannine. In particular, ozone oxidation of cephalomannine in the presence of paclitaxel is a viable initial step to ease the separation of these two similar compounds.[6] The ozonolysis can be controlled to result in complete oxidation of cephalomannine with no or very little oxidative decomposition of paclitaxel, even at 25 °C. The Kingston group briefly examined ozone oxidation of cephalomannine, but not mixtures of paclitaxel and cephalomannine.[7] Kingston subsequently reported methods for selective reaction of cephalomannine in the presence of paclitaxel at the tigloyl amide double bond using osmium tetraoxide[8] or bromine.[9] All methods for separating paclitaxel and cephalomannine described so far require chromatography at some point in the purification.[4,6d,8,9] We describe herein a simple method for cleanly separating these two similar compounds that does not require chromatography.

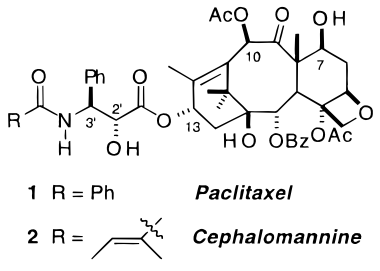
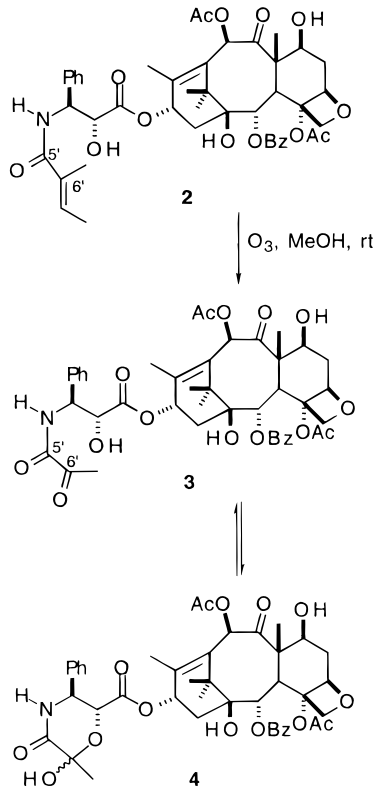


Figure 1.

Scheme 1



The initial step in the current method is oxidation with ozone. The ozonolysis of **2** provides the α-keto amide **3** in good yield (Scheme 1). Under most conditions, addition of a reducing agent, such as dimethyl sulfide, is not required to obtain good yields of **3**. The reducing agent is added to quench potentially explosive peroxides or peroxyacetals. It was initially thought that the 2′-OH group participates in the breakdown of an intermediate ozonide. However, protection of the 2′-OH group with a triethylsilyl group, followed by ozonolysis, quenching, and deprotection did not significantly reduce the yield of **3**. Generally, ozonolysis of α,β-unsaturated carbonyl groups results in complex mixtures of products.[10] Therefore, the ozonolysis of cephalomannine is surprisingly clean, even at 25 °C.

In solution, keto amide **3** is in equilibrium with hemiacetal **4**. ^{13}C NMR resonances that correspond to **4**

(1) Bristol-Myers Squibb announced in early 1995 that paclitaxel produced via a semisynthetic route starting from 10-deacetylbaccatin III was approved by the FDA. All GMP bulk drug for clinical trials and clinical use prior to 1995 was produced via isolation from *Taxus brevifolia* bark.

(2) For recent reviews about taxoid chemistry see: (a) Kingston, D. G. I.; Molinero, A. A.; Rimoldi, J. M. *Fortsch. Chem. Org. Naturst.* **1993**, *61*, 1. (b) Nicolaou, K. C.; Dai, W. M.; Guy, R. K. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 15. (c) *Taxol®: Science and Applications*; Suffness, M., Ed.; CRC Press: New York, 1995.

(3) (a) Powell, R. G.; Miller, R. W.; Smith, C. R., Jr. *J. Chem. Soc., Chem. Commun.* **1979**, 102. (b) Senilh, V.; Blechert, S.; Colin, M.; Guenard, D.; Picot, F.; Potier, P.; Varenne, P. *J. Nat. Prod.* **1984**, *47*, 131.

(4) (a) Rao describes chromatographic methods for separating **1** and **2**: Rao, K. V. Method for the Isolation and Purification of Taxane Derivatives, WO 92/07842, May 14, 1992. (b) Analytical separation of **1** and **2** using HPLC is also difficult. Analytical methods are described in reference 5. For a superior analytical method see reference 5c.

(5) (a) Witherup, K. M.; Look, S. A.; Stasko, M. W.; McCloud, T. G.; Issaq, H. J.; Muschik, G. M. *J. Liq. Chromatogr.* **1989**, *12*, 2117. (b) Cardellina, J. H., III. *J. Liq. Chromatogr.* **1991**, *14*, 659. (c) Richheimer, S. L.; Tinnermeier, D. M.; Timmons, D. W. *Anal. Chem.* **1992**, *64*, 2323.

(6) (a) Murray, C. K.; Beckvermit, J. T.; Ziebarth, T. D. U.S. Patent No. 5,334,732, Aug 2, 1994. (b) Murray, C. K.; Beckvermit, J. T.; Ziebarth, T. D. U.S. Patent No. 5,336,684, Aug 9, 1994. (c) Murray, C. K.; Beckvermit, J. T.; Anziano, D. J. U.S. Patent No. 5,364,947, Nov 15, 1994. (d) Rao, K. V.; Hanuman, J. B.; Alvarez, C.; Stoy, M.; Juchum, J.; Davies, R. M.; Baxley, R. *Pharmaceut. Res.* **1995**, *12*, 1003.

(7) Jitrangsri, C. Ph.D. Thesis, 1986, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

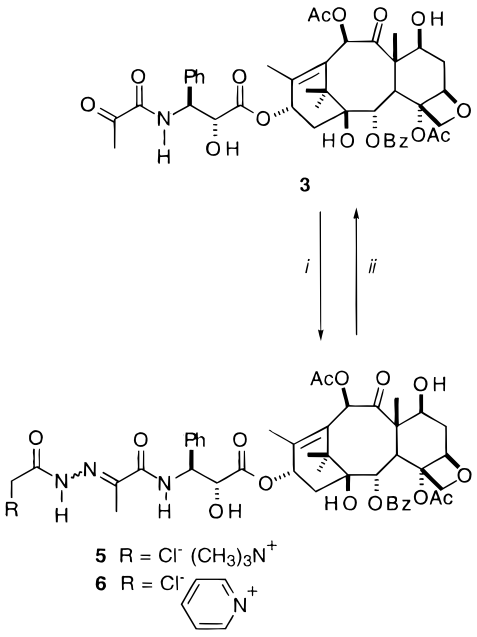
(8) Kingston, D. G. I.; Gunatilaka, A. A. L.; Ivey, C. A. *J. Nat. Prod.* **1992**, *55*, 259.

(9) Rimoldi, J. M.; Molinero, A. A.; Chordia, M. D.; Gharpure, M. M.; Kingston, D. G. I. *J. Nat. Prod.* **1996**, *59*, 167.

(10) (a) Bailey, P. S. Ozonation in Organic Chemistry, Olefinic Compounds; Academic Press: New York, 1978; Vol. 1. (b) Yamamoto, Y.; Niki, E.; Kamiya, Y. *J. Org. Chem.* **1981**, *46*, 250.

S0022-3263(96)01402-8 CCC: $12.00

Scheme 2[a]



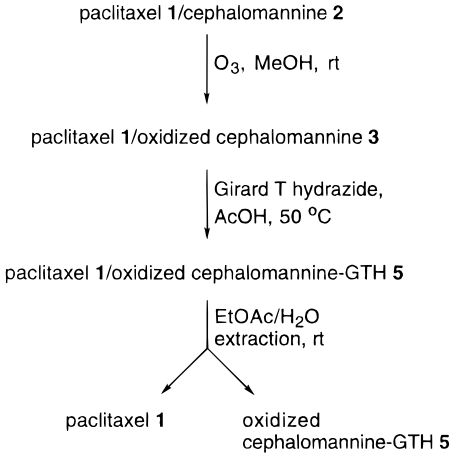
[a] Reagents and conditions: (i) Girard T or Girard P hydrazide, AcOH, 50 °C; (ii) HCl, H_2O, EtOAc, rt.

can be observed in protic solvents. In particular, resonances for the diastereomeric hemiketal carbons are observed in the region of 100 ppm in CD_3OD. Attempts at trapping out the ring-closed ketal by treatment with acidic MeOH were unsuccessful.

Other aspects of the electrophilic activity of the 6′-ketone group of **3** were investigated.[6a,11] Treatment of **3** with Girard T and Girard P hydrazides[12] results in clean formation of hydrazones **5** and **6**, respectively (Scheme 2). Hydrazones **5** and **6** have greatly increased water solubility compared with the starting keto amide **3**. The enhanced water solubility of **5** and **6**, analogs of paclitaxel, makes them interesting; however, bioactivity studies clearly show the potency of these compounds is not greater than that of paclitaxel.[13]

The most attractive characteristics of the water-soluble hydrazones, **5** and **6**, relate to a method for separating paclitaxel and cephalomannine (Scheme 3). Treatment of a 1:1 mixture of paclitaxel and cephalomannine with ozone in methanol results in a mixture of paclitaxel and keto amide **3**. Further treatment of an acetic acid solution of **1** and **3** with Girard T hydrazide at 50 °C for 2 h gives the mixture of **1** and **5**. No decomposition of **1** is observed in the reaction mixture with Girard T hydrazide. Separation of **1** and **5** is most easily achieved by concentrating the mixture to a residue and partitioning between ethyl acetate and water. The recovery of **1** after liquid/liquid extraction is excellent (93%). One or two recrystallizations gives high purity paclitaxel. Hydrazide **5** can be hydrolyzed to yield keto amide **3** by

Scheme 3



treatment with 1.25 N HCl (1 h at rt) in good yield. In fact, purification of **3** from impure mixtures containing cephalomannine is easily achieved by this method.

Many advantages of this method for multigram to kilogram scale separation of paclitaxel from cephalomannine over previously reported chromatographic methods are obvious. In addition, the advantages of using ozone instead of osmium tetraoxide (in stoichiometric or catalytic amounts) are notable. Ozone is less expensive, easier to manipulate in a process, and less toxic with regard to waste streams. Other less obvious advantages include the following: ozone oxidation of cephalomannine in purified mixtures is extremely efficient (usually less than 10 mol equiv are required for complete oxidation of the tigloyl group); the ozone oxidation works well with partially purified or very impure mixtures that contain paclitaxel and cephalomannine;[14] and the oxidation can be done in a variety of solvents and at a variety of temperatures without much loss in selectivity. The main advantage of the process described here is that difficult chromatography can be avoided for purification of paclitaxel from mixtures that contain cephalomannine.[16] Further studies are in progress and will be reported in due course regarding the general application of biomass extract ozonation followed by reaction with Girard reagents or other carbonyl reactive compounds, to effect a nonchromatographic fractionation of complex mixtures of natural products.

Experimental Section[6c]

Oxidized Cephalomannine (3). Cephalomannine **2** (2.66 g, 3.20 mmol) was dissolved in MeOH and treated with 3 equiv of ozone in an oxygen stream at rt. HPLC analysis showed the reaction was complete. The major product, **3**, is best purified by conversion to the hydrazone **5**, followed by liquid/liquid separation and hydrazone hydrolysis (see below).

After the ozonized solution was purged with N_2 and the solvent was removed under vacuum, the dried mixture and 3

(11) A portion of this work was presented at the 208th American Chemical Society National Meeting, Division of Medicinal Chemistry, Poster 65, Washington D.C., Aug. 12, 1994.

(12) Girard reagents have been used extensively for separating mixtures of steroids. (a) Girard, A. *Organic Syntheses;* Wiley: New York, 1943; Collect. Vol. II, p 85. (b) Wheeler, O. H. *Chem. Rev.* **1962**, *62*, 205.

(13) The initial bioactivity studies comprising comparison of tubulin polymerization potency and multiple cancer cell line cytotoxic potency of **5** and **6** relative to paclitaxel were done as described elsewhere.[6b] Complete bioactivity studies including *in vivo* evaluation relative to paclitaxel are pending.

(14) For *Taxus* extracts or semipurified *Taxus* extracts, additional taxanes with accessible olefin groups, for example, taxusin and brevifoliol, are also oxidized by ozone, as well as, lignins and other oxidizable impurities.[15]

(15) (a) For chemical conversions of taxusin see: Miyazaki, M.; Shimuzu, K.; Mishima, H.; Kuragayashi, M. *Chem. Pharm. Bull.* **1968**, *16*, 546. (b) For a chemical conversion of brevifoliol see: Georg, G. I., Cheravullath, Z. S.; Velde, D. V.; Ye, Q. M.; Mitscher, L. A. *Biorg. Med. Chem. Lett.* **1993**, *3*, 1345.

(16) A similar separation of oxidized cephalomannine **3**, and paclitaxel **1**, can be accomplished by stirring an acetic acid solution of the two compounds with a hydrazide reagent bound to a solid phase. Compound **3** is converted to a hydrazone bound to a solid phase and can be filtered away from the paclitaxel remaining in solution.

equiv of Girard T reagent (1.61 g) were dissolved together in AcOH (23 mL). The resulting solution was heated to 50 °C and stirred for 2 h. The solvent was removed under vacuum, and the residue was dissolved in 50/50 EtOAc/H_2O. The two phases were separated, and the aqueous phase was washed until no more impurities were visible by HPLC. The aqueous phase was then acidified with HCl until it was 1.25 N HCl, EtOAc was added, and the biphasic mixture was mixed at rt for 1 h. The two phases were separated and washed. The organic phase was washed sequentially with $NaHCO_3$ (saturated solution) and brine. After drying over $MgSO_4$ and evaporation of the solvent, 1.55 g (1.89 mmol, 59%) of **3** was recovered as a white solid: mp 173−176 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.11 (s, 3H), 1.21 (s, 3H), 1.29−1.58 (m, 2H), 1.64 (s, 3H), 1.78 (s, 3H), 1.89−2.16 (m, 3H), 2.20 (s, 3H), 2.31 (s, 3H), 2.24 (s, 3H), 2.40−2.72 (m, 1H), 3.75 (d, J = 6.8 Hz, 2H), 3.98−4.47 (m, 3H), 4.64 (m, 1H), 4.89 (d, J = 8.5 Hz, 1H), 5.13−5.68 (m, 2H), 5.98−6.20 (m, 1H), 6.25 (s, 1H), 7.27−7.73 (m, 8H), 7.85 (d, J = 9.4 Hz, 1H), 8.07 (d, J = 7.7 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 9.54, 14.65, 20.76, 21.65, 22.51, 24.37, 26.79, 35.62, 35.62, 43.12, 45.72, 55.00, 58.48, 72.04, 72.04, 73.31, 74.96, 75.58, 76.44, 79.00, 81.16, 84.32, 126.97, 126.97, 128.60, 128.60, 128.60, 128.89, 128.89, 129.16, 130.11, 130.11, 133.24, 133.65, 137.14, 141.68, 159.70, 166.88, 170.30, 171.07, 171.93, 195.94, 203.55. The diagnostic signals in CD_3OD of **3** and **4**, including possible solvent addition (CD_3-OD) to the ketone-ketal carbon of **3** and **4** are 97.9 and 197.2 ppm; IR (neat, cm^{-1}) 981.6, 1025.9, 1070.3, 1108.9, 1178.3, 1241.9, 1373.1, 1724.0, 2900.4, 2940.9, 3064.3, 3413.4, 3490.5 cm^{-1}; HRMS (FAB, glycerol/thioglycerol matrix), m/z calcd for $C_{43}H_{50}NO_{15}$ (M^+): 820.3179, measured 820.3168.

Oxidized Cephalomannine Girard T Hydrazone (5). Cephalomannine (526 mg, 0.63 mmol) was dissolved in 45 mL of MeOH (to result in a 0.014 M solution) and treated with 1.9 mmol of ozone in an oxygen stream. The solution was purged with N_2, and the solvent was removed under vacuum. The solid and 0.51 mmol of Girard T reagent were dissolved together in AcOH (5 mL). The solution was heated to 50 °C for 2 h. The solvent was removed under vacuum. The residue was dissolved in equal volumes of H_2O and EtOAc. The phases were separated, and the aqueous phase, containing the hydrazone, was washed extensively with EtOAc to remove any organic soluble impurities. The aqueous phase was then lyophilized yielding the crude hydrazone (431 mg). To obtain an analytically pure sample, 110 mg of the hydrazone was purified by chromatography (SiO_2, 10% H_2O/acetonitrile). The best fraction was concentrated to dryness (71.5 mg, 65% recovery) to yield the white solid hydrazone. An inseparable 10:1 mixture of two hydrazone isomers was isolated; the NMR data is given for the major isomer only: ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.03 (s, 6), 1.50 (s, 3H), 1.64 (m, 1H), 1.78 (m, 1H), 1.79 (s, 3H), 1.95 (m, 1H), 1.98 (s, 3H), 2.12 (s, 3H), 2.24 (s, 3H), 2.32 (m, 1H), 3.35 (s, 9H,), 3.63 (d, J = 7.0 Hz, 1H), 3.98 and 4.02 (d, J = 7.3 Hz, 2H), 4.12 (m, 1H), 4.5 (s, 1H), 4.90 (m, 1H), 4.95 (m, 1H), 5.06 (d, J = 17.2 Hz, 1H), 5.25 (dd, J = 7.6, 7.7 Hz, 1H), 5.43 (d, J = 7.3 Hz, 1H), 5.52 (d, J = 17.2 Hz, 1H), 5.92 (dd, J = 9.1, 9.2 Hz, 1H), 6.30 (s, 1H), 6.39 (d, J = 7.7 Hz, 1H), 7.25 (m, 1H), 7.39 (m, 4H), 7.62 (m, 2H), 7.71 (m, 1H), 7.99 (m, 2H), 8.98 (d, J = 8.4 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 10.38, 12.43, 14.44, 21.28, 21.96, 22.93, 26.93, 35.45, 37.11, 43.54, 46.66, 53.71, 57.01, 57.95, 63.22, 70.09, 70.97, 73.77, 75.08, 75.31, 75.90, 77.36, 80.73, 84.20, 127.83, 128.10, 128.77, 128.92, 129.26, 130.15, 130.53, 133.80, 133.98, 139.97, 146.09, 164.39, 165.78, 167.91, 169.34, 170.53, 172.74, 202.97; IR (neat) 3276, 2964, 1720, 1373, 1246, 1113 cm^{-1}; HRMS (FAB, glycerol/thioglycerol matrix) m/z calcd for $C_{48}H_{61}N_4O_{15}$ (M^+): 933.4132, found 933.4138.

Oxidized Cephalomannine Girard P Hydrazone (6). Cephalomannine (490 mg, 0.59 mmol) was dissolved in 42 mL of MeOH (to result in a 0.014 M solution) and treated with 1.8 mmol of ozone in an oxygen stream. The solution was purged with N_2, and the solvent was removed under vacuum. The solid and 0.47 mmol of Girard P reagent were dissolved together in AcOH (4 mL). The solution was heated to 50 °C for 2 h. The solvent was removed under vacuum. The residue was dissolved in equal volumes of H_2O and EtOAc. The phases were separated, and the aqueous phase, containing the hydrazone, was washed extensively with EtOAc to remove any organic soluble impurities. The aqueous phase was then lyophilized, yielding the crude hydrazone (470 mg). To obtain an analytically pure sample, 275 mg of the hydrazone was purified by chromatography (SiO_2, 10% H_2O/CH_3CN). The best fraction was concentrated to dryness (161 mg, 58% recovery) to yield the white solid hydrazone. An inseparable 6:1 mixture of two hydrazone isomers was isolated, the NMR data is given for the major isomer only: ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.04 (s, 6H), 1.51 (s, 3H), 1.63 (m, 1H), 1.80 (s, 3H), 1.84−1.99 (m, 2H), 2.04 (s, 3H), 2.12 (s, 3H), 2.20 (s, 3H), 2.30 (m, 1H), 3.62 (d, J = 7.3 Hz, 1H), 3.98 and 4.02 (d, J = 8.4 Hz, 2H), 4.11 (m, 1H), 4.75 (dd, J = 7.5, 7.6, 1H), 4.78 (s, 1H), 4.86 (m, 1H), 4.95 (d, J = 7.0 Hz, 1H), 5.32 (dd, J = 7.7, 7.7 Hz, 1H), 5.44 (d, J = 7.3 Hz, 1H), 5.94 (dd, J = 8.3, 8.5 Hz, 1H), 6.30 (s, 1H), 6.45 (d, J = 7.32 Hz, 1H), 6.45 (d, J = 17.2 Hz, 1H), 7.25 (m, 1H), 7.38 (m, 2H), 7.45 (m, 2H), 7.61 (m, 2H), 7.71 (m, 1H), 7.98 (m, 1H), 8.29 (m, 2H), 8.74 (m, 1H), 8.98 (d, J = 8.4 Hz, 1H), 9.18 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 10.39, 12.53, 14.45, 21.28, 21.99, 22.87, 26.95, 35.51, 37.11, 43.57, 46.65, 56.71, 57.94, 62.21, 70.31, 70.95, 73.74, 75.08, 75.31, 75.89, 77.39, 80.75, 84.16, 127.52, 127.87, 128.07, 128.38, 128.82, 129.27, 130.13, 130.51, 133.79, 133.99, 139.95, 146.10, 146.94, 146.95, 164.25, 165.75, 168.73, 169.34, 170.54, 172.82, 202.96; IR (neat) 3265, 2934, 1680, 1493, 1373, 1244, 1072 cm^{-1}; HRMS (FAB, glycerol/thioglycerol matrix) m/z calcd for $C_{50}H_{57}N_4O_{15}$ (M^+): 953.3819, found 953.3818.

Oxidation of a Mixture of Cephalomannine and Paclitaxel with Ozone and Separation Using Girard T Reagent and Selective Precipitation. A mixture of pure paclitaxel (100.6 mg, 0.119 mmol) and cephalomannine (100.5 mg, 0.121 mmol) was dissolved in dry methylene chloride. The mixture was cooled to −78 °C, and 3 mol equiv (compared to cephalomannine) of ozone was bubbled into the solution. After purging the solution with argon the solvent was removed from the mixture. To the dried reaction mixture was added 1.5 mol equiv (compared to the original molar amount of cephalomannine) of Girard T hydrazide reagent (30.8 mg). To dissolve the solids, enough acetic acid was added (880 μL) to make a 0.14 M solution. The reaction mixture was heated to 50 °C with stirring for 2 h. The acetic acid was evaporated and the residue crystallized from MeOH/H_2O. After drying, the recovery of paclitaxel was 79.6 mg (79.1% recovery). The solids were recrystallized from MeOH/H_2O to yield 68.1 mg (67.7% recovery). The chromatographic and spectroscopic data for the isolated compound was identical to a standard sample of paclitaxel.

Oxidation of a Mixture of Cephalomannine and Paclitaxel with Ozone and Separation Using Girard T Reagent and Liquid/Liquid Extraction. An equal mixture of cephalomannine (19.7 mg, 0.024 mmol) and paclitaxel (19.7 mg, 0.023 mmol) was dissolved in 1.2 mL of acetic acid and treated with 2 mol equiv of ozone (relative to cephalomannine). The reaction mixture was purged with argon, the Girard T reagent (20.1 mg) was added, and the solution was heated to 50 °C. After heating for 2 h, the solvent was removed under vacuum. The dried reaction mixture was dissolved in a minimal amount of ethyl acetate and water, and the two phases were separated. The organic phase that contains the paclitaxel was washed sequentially with saturated $NaHCO_3$ and brine solutions. The total residue (19.1 mg) after evaporation was 95% paclitaxel by chromatographic purity analysis with a 93% overall recovery.

Supporting Information Available: Copies of the ^{1}H NMR and ^{13}C NMR spectra for compounds **3**, **5**, and **6** (30 pages). This material is contained in libraries on microfiche, immediately follows this article on the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

JO961402L