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Paclitaxel Stability in Solution

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Research in this laboratory has focused on the cytokinetic effect of taxanes on nonmammalian systems. Taxanes are a class of natural products that includes the well-known anticancer compound, paclitaxel (Taxol). Our methodology for the study of fungal growth in liquid medium amended with paclitaxel included membrane solid phase extraction (SPE) of the fungal broth. This was followed by elution of paclitaxel from the SPE membrane using methanol. The methanolic solution was evaporated under relatively mild conditions, namely 41−43 °C and approximately 85 kPag. Analysis of the concentrated solution indicated that it contained a considerable quantity of 7-*epi*-taxol and smaller quantities of 7-*epi*-10-deacetyltaxol, 10-deacetyltaxol, and baccatin III, in addition to paclitaxel, *even in those cases where the medium had not been inoculated with fungus*. Obviously, fungal metabolism could not account for these observations. Although epimerization in solution at carbon 7 in the C ring of the taxane core has been observed and reported previously, no detailed study of the solution kinetics of paclitaxel degradation, including epimerization, is available. We report here our investigation of the stability of paclitaxel in several solvent systems at various temperatures and pressures. The investigations indicate that the apparent activation energy barrier (E_a) for paclitaxel degradation is highly dependent on experimental conditions. These stability studies emphasize the need to demonstrate explicitly that all taxane degradation, including epimerization, observed during in vitro studies is not an artifact of the analytical methodology employed.

Paclitaxel is a highly functionalized diterpene member of the taxane class of compounds. Initially isolated from the bark of the Pacific yew tree,[1] *Taxus brevifolia*, paclitaxel has since been found in all other known species of *Taxus* in varying quantities throughout the plant.[2−6] Previous research from this laboratory has shown that paclitaxel and other taxanes are present in the needles of many ornamental yews at levels equal to or greater than that found in Pacific yew bark.[6]

Paclitaxel's function in mammals as a mitotic spindle poison, inhibiting cell replication in vitro and in vivo,[7,8] has been extensively documented in the literature. The cytokinetic effect of paclitaxel and other taxanes has also been examined to a limited degree in nonmammalian systems.[9−19] Such investigations with nonmammalian systems may result in novel uses for taxanes, including agrochemical applications.

We have been examining the effect of pure taxanes, as well as a partially purified extract of yew needles, on plant pathogenic fungi.[16,19] The experiments are designed to study (1) the effect of taxanes on the growth and subcellular organization of taxane-sensitive fungi and (2) the fate of paclitaxel and other taxanes exposed to taxane-sensitive versus -insensitive fungi in liquid medium. These investigations required a method to isolate both the fungal mycelium and the taxanes from the medium. Our previously published membrane SPE method[20] appeared to be suitable for taxane recovery. However, during the course of these experiments, it became apparent that paclitaxel degradation was occurring which could not be attributed to fungal metabolism. A detailed study of paclitaxel solution kinetics was necessary in order to interpret our observations.

We report here our studies of paclitaxel stability in several solvent systems under different temperatures and pressures. These kinetics and stability data guided the development of an SPE protocol for isolation of the taxanes from the liquid medium. Since paclitaxel solution stability impacts all in vitro experiments with either mammalian or nonmammalian systems, the data underscore the requirement to demonstrate explicitly that each step of an analytical method used with taxanes not result in the production of artifacts.

EXPERIMENTAL SECTION

Reagents. Organic solvents used for liquid chromatography were Omnisolv grade (EM Science, Gibbstown, NJ); other solvents were HPLC grade (J. T. Baker, Phillipsburg, NJ), as was dimethyl sulfoxide (DMSO, Aldrich Chemical Co., Milwaukee, WI). Tax-

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anes were obtained from the National Cancer Institute (NCI), Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program (Bethesda, MD), with the exceptions of 7-*epi*-taxol, which was received from Bristol-Myers Squibb (Wallingford, CT), and the paclitaxel side chain methyl ester, 3′-(benzoylamino)-2′-hydroxybenzenepropanoate, which was purchased from Hauser Chemical Research (Boulder, CO). The water used was distilled, followed by deionization (DI-DI) in a NANOpure II four-cartridge system (Barnstead/Thermolyne Corp., Dubuque, IA). The fungal medium was prepared from a modified version of Schenk and Hildebrandt's (SH) and autoclaved prior to use.[21]

Apparatus. High-performance liquid chromatography (HPLC) analysis was performed on a Perkin-Elmer (Norwalk, CT) LC250 binary system with modifications to our published HPLC method.[20] Quantitation was performed with PE Nelson 1020 software (v4.71) by the external standard calibration method. The membrane SPE filtration device was either a 47 mm all-glass 1 L filtration apparatus (XX15 047 00, Millipore, Bedford, MA) or a six-position vacuum manifold equipped at each position with a 47 mm glass reservoir and tabulated glass base (98050300809, 3M Corp., St. Paul, MN). Each filtration apparatus was fitted with a Kel-F (3M Corp.) 47 mm support disk. A 47 mm Empore C18 membrane (7460-06, J. T. Baker) was placed on the Kel-F support disk, and a 47 mm poly(propylene) (PP) separator of 10 μm pore size (61757, Gelman Sciences, Ann Arbor, MI) was set on top of the Empore membrane. Kinetics experiments were performed in sealed vacules or ampules (Wheaton Glass, Millville, NJ).

Procedure. *Analytical HPLC.* The mobile phase tubing was maintained at 30−31 °C, while the MetaChem Taxsil SAFE-GUARD guard cartridge (2.0 mm i.d. × 13 mm) and MetaChem Taxsil analytical column (2.0 mm i.d. × 150 mm, 5 μm) were kept at 30 °C. A 5 μL injection of a six-taxane standard, prepared in Omnisolv methanol from the authentic compounds, was made at low and high concentration levels. The low and high level calibration standards contained 0.015−0.050 and 0.65−2.5 μg/μL of each taxane, respectively. The mobile phase consisted of acetonitrile in reservoir A and a 90:10 (v/v) solution of water/methanol in reservoir B. HPLC operating conditions were as follows: a 0.28 mL/min flow rate at 36% A was maintained for 20 min, followed by a linear gradient over 2 min to 65% A, held for 12 min, and a linear gradient to 36% A over 5 min. The final composition was held for 20 min to reequilibrate the system prior to the next injection. The structures of the taxanes of interest, baccatin III, 10-deacetyltaxol, cephalomannine, 7-*epi*-10-deacetyltaxol, paclitaxel, and 7-*epi*-taxol, are provided in Figure 1. These six taxanes are resolved under the specified conditions (Figure 2).

SPE Isolation of Taxanes and Fungal Mycelia. A modification of our SPE method for the large-scale isolation of taxanes from *Taxus* crude extracts[20] was used for collection of the fungal mycelia and taxane/metabolite extraction from the SH medium. The 47 mm PP separator/Empore membrane combination was conditioned prior to sample loading, using ethyl acetate, methanol, and water, successively, to remove contaminants and to solvate the C18 chains in the disk. Following conditioning, 25 mL of water was delivered into the reservoir and passed through at 1 mL/min to ensure that all of the methanol was displaced from the membrane. When ≅10 mL of water remained in the reservoir, the vacuum was released, and the medium/fungal sample, typically ≅15 mL, was added to the reservoir. The vacuum was reestablished and the liquid drawn through the membrane until it was just dry. Water (20 mL) was delivered into the reservoir to wash off any polar compounds from the fungal mat and from the Empore membrane. The vacuum was maintained for 5 min to remove thoroughly residual water from the SPE membrane and the support disk. The mycelium and PP separator were removed. Methanol (15 mL) was then delivered into the reservoir above the Empore membrane, and, with the vacuum in place, the methanol fraction was collected, transferred to a round-bottom flask, and concentrated according to one of the two procedures detailed below. Data from the fungal growth studies will be published elsewhere.

Concentration Procedure 1. The methanol fraction was concentrated to dryness in a water bath at 41−43 °C by rotary evaporation at reduced vacuum (85 kPag), established with water aspiration for 20 min. The resulting residue was reconstituted using sonication in methanol to a final volume of 2 mL for analysis by HPLC.

Concentration Procedure 2. The methanol fraction was concentrated to ∼0.5 mL on a rotary evaporator at high vacuum, established with a Duo Seal vacuum pump (Welch Manufacturing Co., Chicago, IL). The sample was kept in a water bath at ambient temperature (∼21 °C) during evaporation. Solvent reduction required 6−7 min, after which the sample was reconstituted with methanol to 2 mL and analyzed by HPLC.

Kinetics of Paclitaxel Degradation in Solution. We investigated paclitaxel degradation kinetics in DMSO, SH medium, Omnisolv methanol, chloroform, 1:1 (v/v) SH medium/DMSO, and 1:1 (v/v) SH medium/methanol. Stock solutions of paclitaxel in DMSO, methanol, or chloroform were prepared as needed, typically at 1.5 μg/μL. The ampule or vacule was charged with 10 μL of appropriate paclitaxel stock solution and 490 μL of the solvent under study.

Sealed ampules or vacules containing the paclitaxel solution were placed in a constant temperature bath and removed at selected times for HPLC analysis. During the course of the experiments, the DMSO and SH solutions generally changed from colorless to orange/dark brown.

RESULTS AND DISCUSSION

Kinetics Studies. It has been reported that paclitaxel converts primarily to 7-*epi*-taxol, the thermodynamically more stable isomer,[22] upon heating in the dry state,[23] in organic solvents,[22−25] and in cell culture medium.[26] It is reasonable that 7-*epi*-taxol is the thermodynamically more stable isomer due to hydrogen bonding between the C7α-OH and C4α-acetate acyl oxygen.[25] Epimerization at C7 is reversible and proceeds via a proposed[24] retroaldol/aldol intramolecular mechanism. The proposed pathway requires transfer of the hydroxyl hydrogen from C7 to the C9 carbonyl with concomitant aldehyde formation at C7, ring cleavage between C7 and C8, double-bond formation between C8 and C9, and hence enol formation at C9. Free rotation

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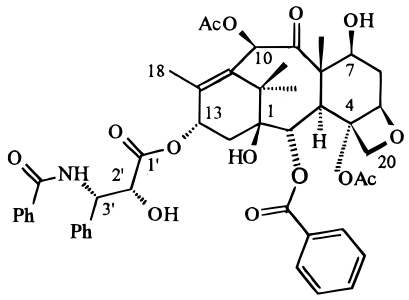
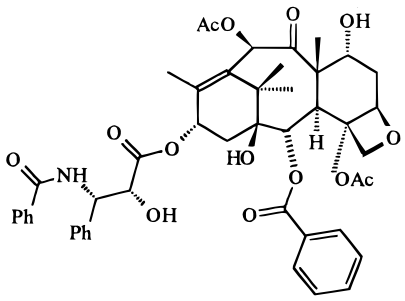
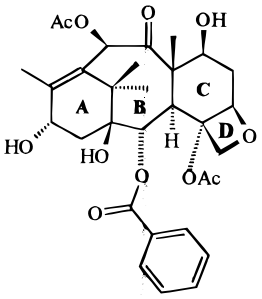


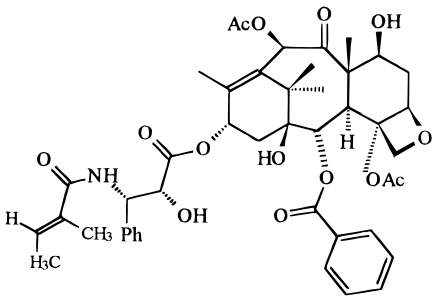
Paclitaxel



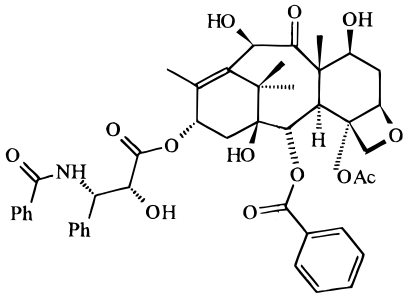
7-*epi*-Taxol



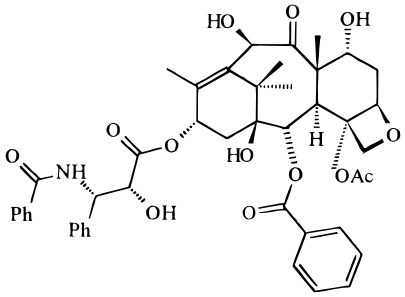
Baccatin III



Cephalomannine



10-Deacetyltaxol



7-*epi*-10-Deacetyltaxol

Figure 1. Taxane structures. Ph, phenyl; OAc, acetate.

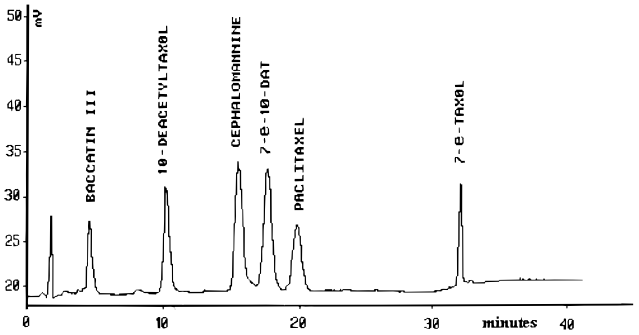


Figure 2. Chromatogram of six-taxane standard on 2.0 mm i.d. × 150 mm MetaChem Taxsil column under elution conditions described in the Analytical HPLC section.

about the single bond between C6 and C7 permits the electron density in the C8−C9 alkene to attack the aldehyde carbonyl on either face of the C7 carbon, forming either paclitaxel or 7-*epi*-taxol. Proximity of the C7 and C9 moieties of the taxane core is essential to the intramolecular mechanism and has also been proposed to account for intramolecular acyl migrations between these two positions observed under mild acid catalysis of taxanes isolated from *T. brevifolia*.[27]

We have studied the kinetics of paclitaxel degradation in solvent systems corresponding to those in our fungal experiments or to the possible solvent composition remaining under evaporation. Paclitaxel stability was also studied in chloroform since epimerization in this solvent has been reported.[24]

Typical first-order kinetics, as represented in Figure 3 for paclitaxel decomposition in 1:1 (v/v) SH media/DMSO, was generally observed in all solvent systems studied. The first-order or pseudo-first-order rate constant, k, at a given temperature, T, for a particular solvent system was determined according to

$$\log [\text{paclitaxel}]_t = \frac{-k}{2.303}t + \log [\text{paclitaxel}]_0 \qquad (1)$$

where $[\text{paclitaxel}]_t$ is the paclitaxel concentration at time t and $[\text{paclitaxel}]_0$ is the paclitaxel concentration at $t = 0$.

The observed rate constants were used in the Arrhenius equation,

$$\log k = \frac{-Ea}{2.303RT} + \log A \qquad (2)$$

to calculate an apparent E_a for paclitaxel degradation as a function

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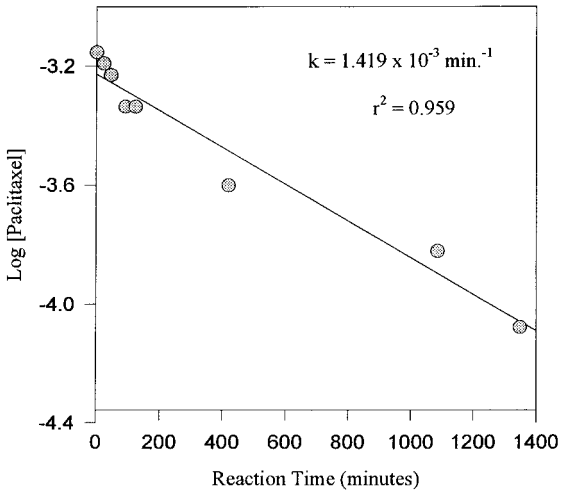

Figure 3. Typical first-order plot of degradation of paclitaxel. Plot of log [paclitaxel] vs time for paclitaxel degradation in 1:1 (v/v) SH media/DMSO at 115 ± 2 °C.

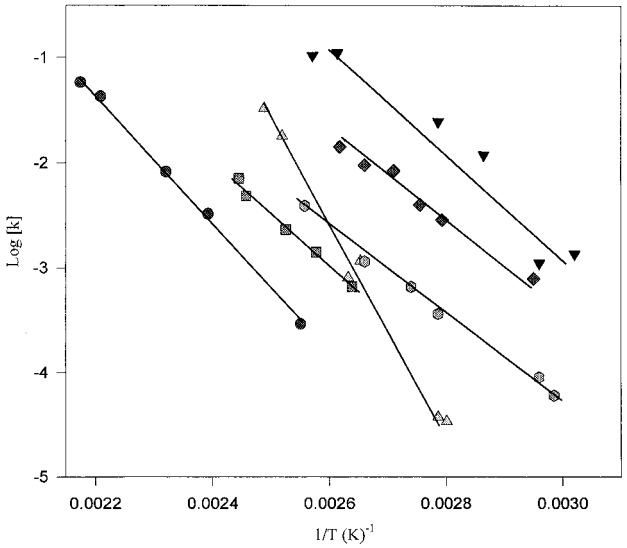

Figure 4. Arrhenius plot for paclitaxel degradation in different solvent systems.

of the solvent. The data from the kinetics experiments are summarized in Table 1 and Figure 4. Included in Table 1 are the E_a values for paclitaxel degradation in isobutyl alcohol and in the dry state which we calculated from ref 23 to equal 132 and 129 kJ/mol, respectively. Although these two E_a values are similar, the authors report that degradation is 5−6 times faster in isobutyl alcohol than in the dry state.

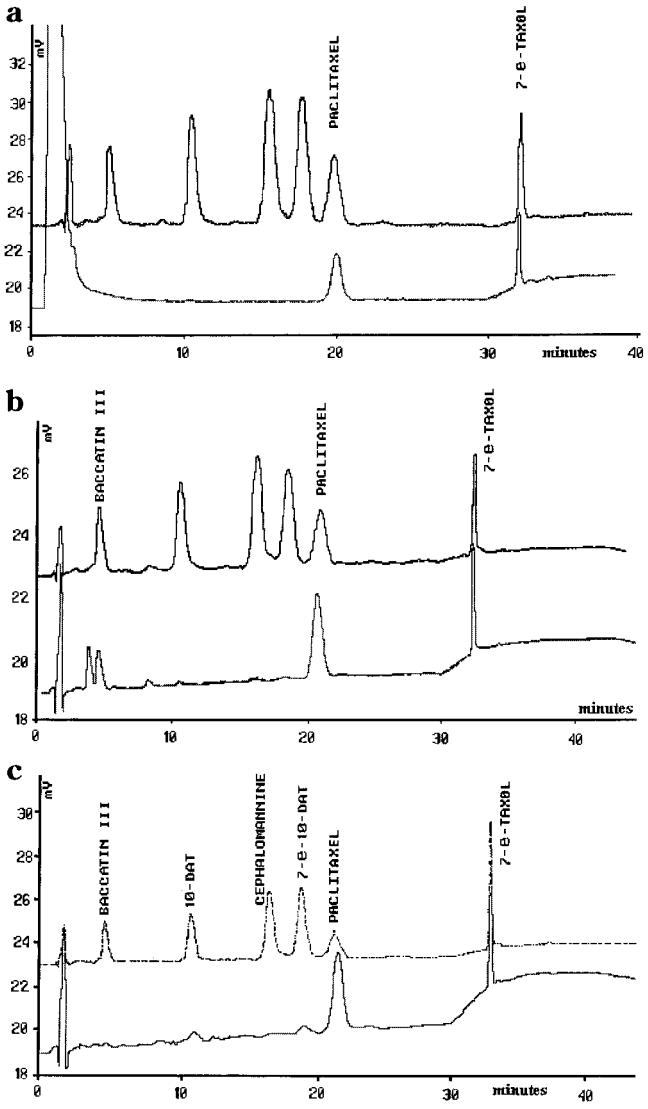

Figure 5. (a) Paclitaxel degradation in SH medium at 86 °C and t = 20 176 min, overlayed with corresponding six-taxane standard; analysis performed on 2.0 mm × 150 mm column. (b) Paclitaxel degradation in methanol at 116 °C and t = 10 min, overlayed with corresponding six-taxane standard; analysis performed on 2.0 mm × 150 mm column. (c) Paclitaxel degradation in methanol under concentration procedure 1 conditions; analysis on 2.0 mm × 150 mm column column, overlayed with six-taxane standard.

Table 1 and Figure 5 provide information regarding the primary degradation products observed in our kinetics studies. While epimerization is the primary route of degradation in SH medium, as shown in Figure 5a, several competing degradation routes are

Table 1. Kinetics of Paclitaxel Degradation in Solution

solvent system	boiling point (°C)	temperature range studied (°C)	E_a (kJ/mol)	$t_{1/2}$ at 43 °C	primary degradation products
SH	≅100	84−129	186	149 y	7-*e*-T
DMSO	189	119−187	118	28 y	7-*e*-T
MeOH Omnisolv	62	58−116	89	1.8 d	7-*e*-T, Bac III, MESC[a] + others
1:1 (v/v) SH/DMSO		105−136	97	0.9 y	7-*e*-T
1:1 (v/v) SH/MeOH		62−118	78	42 d	7-*e*-T, Bac III, MESC[a] + others
$CHCl_3$	60.9	66−109	74	4.1 d	high t_R compounds[b] + 7-*e*-T (at elevated temps)
isobutyl alcohol	108	80−120	132[c]	29 y	7-*e*-T
dry		80−120	129[c]	137 y	7-*e*-T

[a] 7-*epi*-Taxol, baccatin III, and paclitaxel methyl ester side chain at C13 respectively, see text. [b] Late-eluting compounds that are most likely taxanes. [c] Calculated from ref 23.

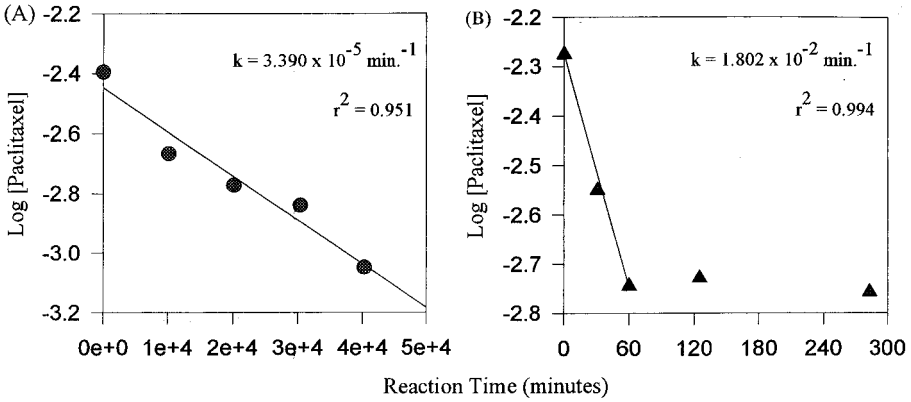

Figure 6. Linear and biphasic first-order paclitaxel degradation kinetic plots in SH medium at (A) 86 and (B) 124 °C.

Table 2. Paclitaxel Stability under Different Experimental Conditions

	solvent system	concentration procedure	recovery of spiked paclitaxel (%)	degradation products	duration of concentration (min)
A	crude extract[a]	1	100.8−110.0 (n = 4)	none detected	20−30
B	MeOH fraction from fungal medium[b]	1	24.0−122.1 (n = 12)	7-*e*-T, 7-*e*-10-DAT, 10-DAT, Bac.III[c]	15−20
C	CH_3OH, HPLC or Omnisolv grade	1	14.3−102.9 (n = 9)	7-*e*-T, 7-*e*-10-DAT, 10-DAT, Bac.III	15−20
D	80% taxane fraction[d]	1	86.4−108.3 (n = 10)	none detected	15
E	MeOH fraction from fungal medium[b]	2	73.6−108.7 (n = 35)	none detected	6−7
F	CH_3OH, HPLC grade	2	93.2−105.1 (n = 5)	none detected	6−7

[a] See ref 20. [b] See text. [c] 7-*epi*-Taxol, 7-*epi*-10-deacetyltaxol, 10-deacetyltaxol, and baccatin III, respectively. [d] Fraction from SPE cleanup of crude *Taxus* needle extract eluted with 80% methanol/20% water.[20]

operative in Omnisolv methanol (Figure 5b). As shown in Figure 5b, in addition to 7-*epi*-taxol (retention time (t_R) = 33 min) and baccatin III (t_R = 4.6 min) formation, there is a polar compound with t_R = 3.8 min. Use of an authentic standard indicated that this peak corresponds to the methyl ester of the paclitaxel side chain at C13.

It is noteworthy that we observed minimal epimerization in $CHCl_3$. In this solvent system, the principal degradation products were unidentified taxanes (λ_{max} = 230 nm), with small amounts of 7-*epi*-taxol formed at elevated temperature. This is in contrast to the work of McLaughlin et al., who report that epimerization in chloroform was observed upon prolonged standing.[24] No additional details are provided in this citation.

Although in SH medium paclitaxel degradation at lower temperatures, i.e., 84, 86, and 107 °C, was first order throughout the reaction time investigated, at elevated temperatures, namely 124 and 129 °C, degradation was biphasic (see Figure 6). The first portion of the plot of log [paclitaxel] vs time at each of the higher temperatures was linear with a negative slope; the second portion of the plot was also linear but with slope ≅ 0. To construct the Arrhenius plot for paclitaxel degradation in SH medium, the first portion of the pseudo-first-order plot was used at those reaction temperatures exhibiting biphasic kinetics.

Paclitaxel Stability. Most procedures for quantitation of taxanes from *Taxus* tissues begin with methanol or methylene chloride extraction,[1−6,28,29] which solubilizes many organic compounds present in the plant tissues. These crude taxane extracts are concentrated, typically by rotary evaporation, and reconstituted in a much smaller volume of solvent, followed by either direct analysis or chromatographic purification and then analysis.

It is an implicit requirement of the analytical methodology that taxanes of interest not be artifacts of the extraction, concentration, or purification steps. In our laboratory, when paclitaxel is spiked into a crude methanolic extract of *Taxus* needles, concentrated under the conditions specified in concentration procedure 1, and partially purified by SPE, no degradation of paclitaxel is observed (Table 2, row A). However, in the workup of the control flasks from our fungal studies, containing only paclitaxel (in DMSO) and SH fungal medium incubated at 25 °C, quantitative recovery of paclitaxel from the medium was problematic (Table 2, row B). For most flasks, paclitaxel recovery was less than 100%, and varying amounts of 7-*epi*-taxol, baccatin III, 7-*epi*-10-deacetyltaxol, and 10-deacetyltaxol were also noted. Consistent with these observations, we subsequently found that, when pure paclitaxel in methanol, either HPLC or Omnisolv grade, was evaporated at 41−43 °C and 85 kPag, variable and nonquantitative amounts of paclitaxel were recovered, together with 7-*epi*-10-deacetyltaxol, 7-*epi*-taxol, baccatin III, and 10-deacetyltaxol (Table 2, row C). This is in direct contrast to the 100% recovery of paclitaxel spiked into crude methanolic extracts of *Taxus* needles described previously.

Extrapolation of each Arrhenius plot for paclitaxel degradation to 43 °C, the bath temperature employed in concentration procedure 1, generates a rate constant from which the degradation half-lives, $t_{1/2}$, can be calculated. These values are summarized in Table 1 and vary considerably depending on the solvent system. The calculated $t_{1/2}$ value for degradation in methanol predicts far less paclitaxel degradation at 43 °C than is actually observed following rotary evaporation either of samples from our fungal experiments or of pure paclitaxel in methanol (see Table 2, rows B and C). Therefore, solution composition, solution pH, reduced pressure, contact with glass surfaces, and/or trace amounts of catalyst in the solvent are among the possible factors affecting paclitaxel degradation in methanol under rotary evaporation conditions. Reduced pressure may enhance paclitaxel degradation by removing the relatively volatile methyl ester side chain from the solution (compare Figure 5b with Figure 5c). Indeed,

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although neat Omnisolv methanol was used to study paclitaxel degradation kinetics (see Figure 5b) and paclitaxel stability under concentration procedure 1 (see Figure 5c), qualitative and quantitative differences in the taxanes produced in each of these experiments are noted. It was beyond the scope of the present research to ascertain which factor(s) was responsible for the considerable degree of paclitaxel degradation observed with concentration procedure 1.

To account for the reduced paclitaxel stability observed under rotary evaporation conditions, several modifications in our laboratory procedure were necessary. Changes incorporated into concentration procedure 2 include a reduction in the temperature of evaporation, from 43 °C to ambient temperature (~21 °C), and decreased time of exposure to reduced pressure, from 15 to 7 min. To minimize the rotary evaporation period, we have determined that it is necessary to maintain a vacuum on the Empore membrane/47 mm Kel-F disk for at least 5 min after the 20 mL water wash so as to remove as much water as possible prior to collection of the 100% methanol fraction. The 47 mm Empore membrane and 47 mm Kel-F support disk have a combined volume of approximately 7−8 mL, and this volume of water can be transferred to the methanol fraction upon elution of the disk. This added step in the SPE elution sequence ensures that the collected methanol fraction has a solvent composition that is consistent from sample to sample and contains minimal water. Larger volumes of water in the methanol fraction will require longer time under vacuum. This, in turn, increases the possibility of paclitaxel degradation. The incorporation of this drying step, combined with the reduced time and temperature for solvent reduction in concentration procedure 2, eliminates paclitaxel degradation in methanol (Table 2, rows E and F).

Once an appropriate concentration procedure was devised, consistent quantitative recovery of paclitaxel from control flasks containing aqueous SH medium was achieved. It is also worth noting that we have not observed adhesion of paclitaxel to the glass surfaces in any of these flasks, as reported by Song et al.,[30] although the paclitaxel concentration, the organic solvent concentration, and the duration of the experiments in the two laboratories are comparable.

CONCLUSIONS

Our observation of variable paclitaxel degradation under relatively mild solvent reduction conditions necessitated this study of the solution stability of the compound. We determined that the degradation of paclitaxel in a variety of solvent systems followed first-order kinetics. The principal degradation product was 7-*epi*-taxol, although this observation was temperature dependent in some solvents. The notable exception was chloroform, in which epimerization was minimal. In most solvent systems, 7-*epi*-taxol formation increased during the initial stages of the reaction, reached a maximum, and then decreased, while the paclitaxel concentration linearly decreased according to the first-order rate equation.

From our kinetics experiments, we have found that paclitaxel is most stable in SH (aqueous) medium, followed by DMSO, and 1:1 (v/v) SH/DMSO. It is least stable in methanol. The kinetics data permitted us to calculate the half-life of degradation in methanol expected at the temperature previously used by us for evaporation under reduced pressure, 43 °C. Comparison of the calculated $t_{1/2}$ with the observed degradation indicated that paclitaxel solution stability depends on several experimental conditions and cannot be predicted solely on the basis of temperature. We hypothesized above on potential factors that might impact paclitaxel degradation during solvent reduction. For example, trace impurities in the methanol may be concentrated during solvent reduction of pure solutions of paclitaxel in methanol, enhancing the effect of catalysts, such as hydroxide and metal ions. The data from the paclitaxel spike recovery experiments suggest that other components present in the crude *Taxus* needle extract "protect" the taxanes from enhanced degradation during evaporation (see Table 2, row A). These protective nontarget compounds appear to be carried over into the 80% methanol/20% water fraction from SPE cleanup of the crude needle extract (see Table 2, row D), attenuating any catalytic effects. However, when methanolic solutions of paclitaxel containing 17%−30% water, but lacking these nontarget compounds, are concentrated using concentration procedure 1, formation of 7-*epi*-taxol and other taxanes was observed (data not shown). Association of paclitaxel with these coextractives from *Taxus* needles may render the molecule less vulnerable to degradation. This proposal is similar to that of Ringel and Horwitz, who suggested that paclitaxel bound to microtubles is less susceptible to modification.[26]

Our studies underscore the necessity for the analyst to demonstrate explicitly that each step in the methodology employed for the in vitro study of taxanes does not result in artifact production. In the research underway in our laboratory, special concentration conditions must be utilized that eliminate paclitaxel degradation in methanol and allow for recovery, identification, and quantitation of the taxanes from the fungal medium. In other laboratory studies, different precautions will be appropriate.

The paclitaxel standard received from NCI contained a small amount of cephalomannine. Like paclitaxel, cephalomannine degradation followed first-order degradation kinetics. However, cephalomannine degradation products were not investigated in these studies.

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