20-*O*-Acylcamptothecin Derivatives: Evidence for Lactone Stabilization

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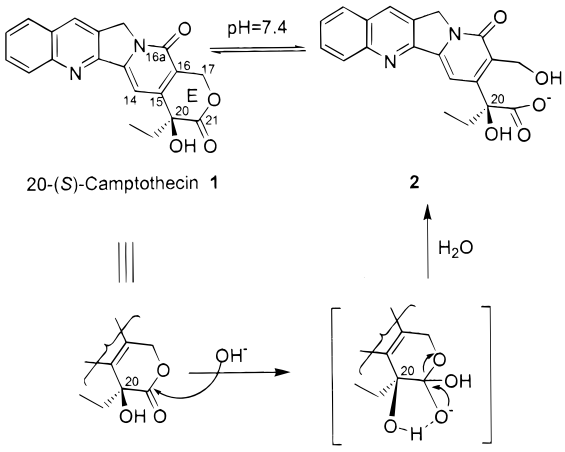
Convincing UV and NMR spectrophotometric evidence is presented which demonstrates that at physiological pH, 7.4, 20-*O*-acyl derivatives of camptothecin (CPT) are substantially more stable in the lactone form than the 20-OH parent. Additionally, it was determined by HPLC analysis that the lactone ring of a 20-*O*-ether derivative of CPT underwent endocyclic ring opening at pH ≥ 8.5, while the lactone ring of 20-*O*-acyl CPT derivatives remained unaffected. PEG (and other smaller alkyl) 20-*O*-acyl-CPT derivatives released native CPT at pH > 9.5, which arises from exocyclic cleavage, thus precluding isolation of any open CPT acyl PEG (or alkyl) carboxylate forms.

Introduction

The isolation and structure determination of 20(*S*)-camptothecin (CPT, **1**), a pentacyclic alkaloid derived from the Asian tree *Camptothica acuminata*, was first accomplished by Wall et al. in 1966.[1] Due to its remarkable efficacy in animal models, this compound was heralded as one of the most important anticancer drugs of the decade, but in human clinical studies held in the late 1960s disappointing results were reported. In those studies the open acid form of CPT was employed as its water soluble sodium salt, $CPT^- Na^+$ (**2**), but produced few or no beneficial effects to cancer patients while bone marrow and nonhematologic toxicities were often encountered.[2] Attempts at improving the aqueous solubility of CPT while maintaining the neoplastic activity of the compound eventually resulted in the development of the water soluble 10-OH CPT analogues, Irinotecan,[3] and Topotecan,[4] both of which were approved for human use in the U.S. in 1997.

Research studies conducted after the early clinical studies of $CPT^- Na^+$ have demonstrated that the lactone ring (E-ring) as well as the 20-OH group of camptothecin are critical for antitumor activity, and that antitumor activity correlates with topoisomerase I inhibition.[5−8] The mechanism whereby CPT exerts its anticancer effect has not been fully ascertained although Wall[9] and Nicholas[10] have clearly shown that the 20-OH group of the E-ring lactone is necessary for the opening of the lactone ring. Furthermore, $CPT^- Na^+$ was also demonstrated to be 10-fold less active *in vivo*,[11,12] and in cell cultures had a lower therapeutic index in the P388 murine model than the intact lactone form.[11]

Scheme 1



Fassberg and Stella[13] have postulated that CPT lactone ring opening, arising from nucleophilic attack at the acyl carbon, probably involves the 20-OH group in one of the proton-transfer steps or in stabilizing the transition state perhaps via a strong intramolecular H-bond as shown in Scheme 1. Recent studies in human plasma (pH 7.4, 37 °C) have demonstrated that CPT lactone rapidly

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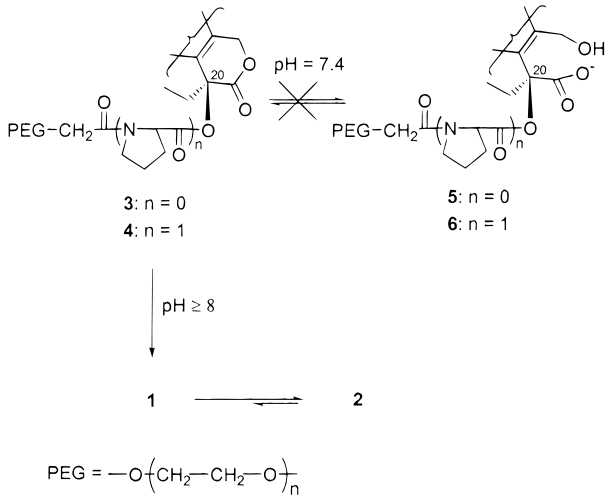
Scheme 2



converts to the carboxylate form ($t_{1/2} = 11$ min).[14,15] Using plasma and protein solutions, Burke and Mi[14,15] have extensively studied the protein binding of the two forms of CPT and several water soluble analogues *in vitro*. They determined that binding of CPT preferentially occurs for the carboxylate form. This has the effect of shifting the equilibrium between the lactone and carboxylate forms in favor of the latter one: in solutions of human plasma albumin (HSA), buffered at pH 7.4, the open form accounts for >99% of the drug. We have recently shown[16] that application of a poly (ethylene glycol) (PEG) prodrug strategy carried out by conjugation of CPT directly to PEG diacid (molecular weight 40000), results in a highly water soluble delivery system for CPT parenteral administration. We also presented HPLC experimental data to support our premise that esterification of the 20-OH of CPT stabilized the lactone ring, a phenomenon previously overlooked.[17] The enhanced E-ring stability and solubility afforded by PEG-CPT derivatives undoubtedly accounts for the exceptional activity observed for this class of compounds.[18] Tumor accumulation of **3**,[16] for example, by the EPR effect[19] will now result in the interstitial enzymatic exocyclic cleavage of the PEG ballast freeing CPT in its active lactone form. CPT represents about 1.7 wt % of the water soluble PEG conjugates **3**, thereby enabling aqueous incubation of the PEG ester (prodrug) with HSA at pH 7.4. Under these conditions no binding to the protein was observed, and we believe this constitutes indirect proof that the integrity of the lactone ring has been maintained (Scheme 2). To determine if possible interactions with HSA could occur with a pegylated open form of CPT, attempts were made to isolate the open carboxylate PEG prodrug form (**5**) at higher pH (8.0), but these were unsuccessful: only native CPT in its open form could be detected. In a preliminary analysis it was assumed that ester hydrolysis occurred first, and the CPT E-ring then underwent rapid opening. Continued interest in PEG-CPT derivatives, one of which is currently in Phase I clinical trials, has led us to carry out additional experimentation in order to gain further insight into the chemistry of the CPT lactone ring. A greater understanding of the factors effecting the stability, and ultimately the hydrolytic process, could be of benefit in the design of more efficacious clinical candidates.[18]

In the present study we hoped to achieve two goals. The first was to demonstrate conclusively that at physiological pH, 20-*O*-acyl derivatives of CPT were indeed more stable in the lactone form than the 20-OH parent by using NMR and UV detection. The second was to establish the extent of acyl stabilization at higher pH using novel CPT 20-*O*-acyl derivatives, and to eventually differentiate whether exo- or endocyclic hydrolysis occurs at the E-ring at a predetermined pH. If conditions for isolation of an open PEG acid form were determined it would result in a potential new prodrug form of CPT. The results of this study provided novel insights into the inordinate stability of the CPT lactone E-ring, and clearly implicated exocyclic hydrolytic cleavage of 20-acyl derivatives of CPT as the mechanism whereby CPT is generated.

Chemistry

For NMR and UV studies, PEG-20-*O*-Pro-CPT (**4**), a water-soluble ester was synthesized and found to have a much longer half-life than the simple PEG-CPT ester, **3**, in pH 7.4 PBS buffer at 37 °C (900 h vs 27 h). We felt that the extended half-life of **4** was a desirable feature since it would enable equilibrium measurements to be done over a longer period of time, and thus increase the opportunity of observing any small amount of open form **6** that might result (Scheme 2). Other, more stable acyl CPT derivatives, viz. PEG-20-*O*-carbamate (**8**) and carbonates (**9** and **10**) (Scheme 3), and a nonacyl CPT derivative (**12**) were also synthesized for this purpose (Scheme 4).

CPT reacted smoothly with triphosgene in the presence of DMAP in dichloromethane (DCM) to generate, in situ, the highly reactive intermediate chloroformate **7** in almost quantitative yield (by HPLC). Compound **7** reacted readily with PEG-amine to give the corresponding PEG-20-*O*-carbamate **8**. Reaction of PEG-OH and methanol with **7** under similar conditions afforded the CPT carbonate derivatives **9** and **10** respectively. Using a convenient procedure developed by Yamada[20] for alkylation of simple 3° alcohols, the relatively stable ether derivative **12** was prepared in 83% yield by the facile reaction of CPT with acetic anhydride and dimethyl sulfoxide (Scheme 4). The hydrolysis of these new compounds was studied at different pH's and temperatures. Representative open ring models $CPT^{-}\ Na^{+}$ (**2**),[21] and the benzylamide (**11**) derived from ring opening of CPT with benzylamine[22] (Scheme 4) were prepared by published procedures for NMR studies.

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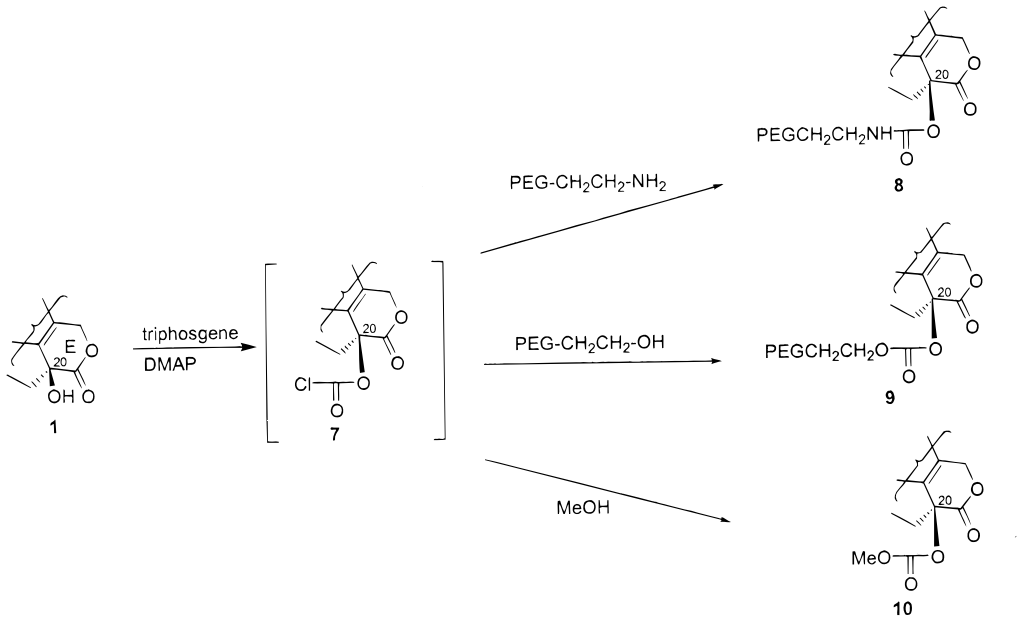
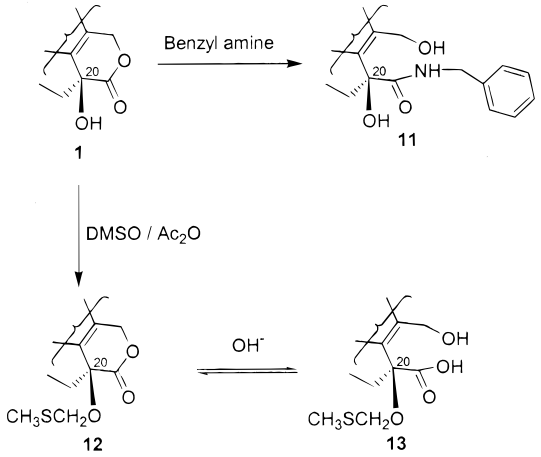
Scheme 3



Scheme 4



Results and Discussion

NMR Studies. Table 1 presents the significant carbon and proton resonance of rings D and E relevant to the open and closed lactone forms of CPT (**1** and **2**) and CPT derivatives **4** and **11**. As indicated in the table, there are significant differences between the carbon resonance in the open carboxylate and closed lactone forms of the E ring which allow the clear identification of these forms in a mixture. For example, in **2** and **11** where the E-ring exists in the open carboxylate form, the ^{13}C NMR spectra clearly shows that the chemical shift for C-17 in both compounds moves more than 9 ppm upfield, while the chemical shift for C-20 moves about 8 ppm downfield comparing to those in native CPT **1**. There were also significant changes in the chemical shifts for C-14, C-15, C-16, C-16a, C-21, etc. For both **2** and **11**, H-17 is directly affected by lactone opening and closing. In the ring-opened compound **2**, the chemical shift is 0.7 ppm upfield comparing to **1**, while for **11** a similar shift of 0.3 ppm is observed.

The stability of **4** was studied initially in order to assess whether ring opening occurs at physiological pH. Both ^{13}C and ^{1}H NMR spectra for **4** are in agreement with those observed for **1,** which has a closed lactone E-ring. ^{13}C chemical shifts for C-14, C-15, C-16, C-16a, C-17, C-20, C-21 of **4** are all very similar to those in **1**, and ^{1}H chemical shifts for H-17 are identical for both **1** and **4**.

After 24 h incubation of **4** in PBS buffer the NMR spectra of the organic material isolated using two different procedures (see Methods) were identical to compound **4**. Thus, no evidence was obtained to indicate any open form **6** was produced at pH 7.4.

UV Studies. UV spectrophotometry was also used to monitor the stability of the lactone ring of **4** at pH 7.4. The characteristic UV wavelength absorption between the native CPT lactone form (λ_{max}: 355 nm) and the CPT carboxylate form (λ_{max}: 363 nm)[23] were sufficiently different to be used as a diagnostic tool. It was assumed that similar shifts in wavelength would also occur for **6**, the carboxyl form of PEG-20-*O*-Pro-CPT, which would convincingly demonstrate the establishment of an equilibrium if that were the case for 20-acylated CPT derivatives.

The maximum UV absorption wavelength of native CPT (lactone form) and **4** in acidic media (pH 3.5) was determined to be 355 nm (Figure 1). A shift in maximum absorption wavelength from 355 to 363 nm was observed for CPT during the 24 h incubation at pH 7.4 and 37 °C (physiological conditions, Figure 2). However, the maximum absorption wavelength of **4** (which exhibits an UV profile similar to that of the lactone form of CPT at pH 3.5, Figure 1) remained the same at 353 nm over the 24 h incubation period at 37 °C and pH 7.4 (Figure 2).

A kinetic profile (Figure 3) clearly shows the stability of **4** under physiological conditions. No evidence was found to substantiate formation of any pegylated open form (**6**).

These results support the NMR data, and clearly demonstrate that the lactone ring of ester **4** is stable under physiological conditions, and are in contrast to the unsubstituted CPT lactone, which is labile, and undergoes rapid hydrolysis under identical conditions.[14, 15]

Hydrolysis Studies. The stability of the E-ring in the water soluble PEG 20-acyl CPT derivatives was also

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Table 1. ^{13}C and ^{1}H NMR Resonance (ppm) for CPT and Its Derivatives[a]

compd	C-14	C-15	C-16	C-16a	C-17	C-20	C-21	H-17
20-(*S*)-CPT (**1**)	96.6	149.9	119.0	156.7	65.2	72.3	172.4	5.4
$CPT^{-}\ Na^{+}$ (**2**)	100.1	153.0	127.0	162.0	56.0	80.0	175.0	4.7
PEG-20-*O*-proline CPT (**4**)	96.5	148.3	118.7	156.8	66.4	76.1	170.7	5.4
CPT-benzyl amide (**11**)	99.4	152.7	126.0	160.9	55.6	79.5	173.0	5.1

[a] All spectra were obtained in $CDCl_3$ with the exception of **2**, which was obtained in a 50:50 (v/v) mixture of $CDCl_3$ and CD_3OD.

Table 2. Results of a Hydrolysis Study of 20-(*S*)-Camptothecin Derivatives

	pH = 7.4		pH = 8.0		pH = 8.5		pH = 9.0		pH = 9.5	
compd	% of carboxylate form	% of free CPT	% of carboxylate form	% of free CPT	% of carboxylate form	% of free CPT	% of carboxylate form	% of free CPT	% of carboxylate form	% of free CPT
PEG-Pro-CPT (**4**)	0	8.8	0	10.2	NA[a]	NA	0	35	NA	NA
PEG-NHCOCPT (**8**)	0	1.9	0	4.6	NA	NA	0	10.6	NA	NA
PEG-OCOCPT (**9**)	0	3.1	0	6.9	NA	NA	0	13.8	NA	NA
$CH_3OCOCPT$ (**10**)	0	0	0	0	0	0	0	1.2	0	13
CH_3SCH_2OCPT (**12**)	1.5	0	2.0	0	4.5	0	15.0	0	45.0	0

[a] NA: not available.

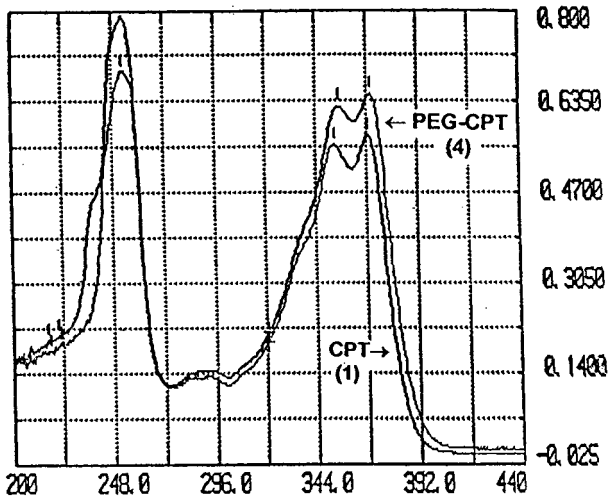


Figure 1. UV spectra of CPT (**1**) and PEG-CPT (**4**) in pH 3.5 buffer after 5 min at 37 °C.

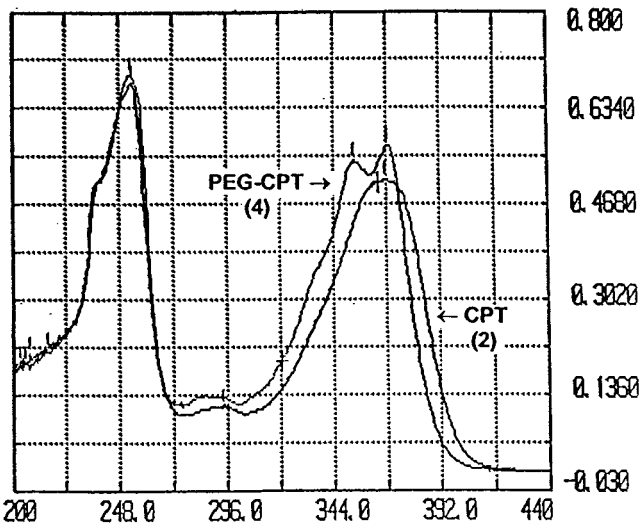


Figure 2. UV spectra of CPT (**1**) and PEG-CPT (**4**) in pH 7.4 buffer after 24 h at 37 °C.

studied employing buffer hydrolysis at different pH's: analysis of the reaction mixtures was carried out using reversed phase HPLC (RP-HPLC) and the results shown in Table 2.

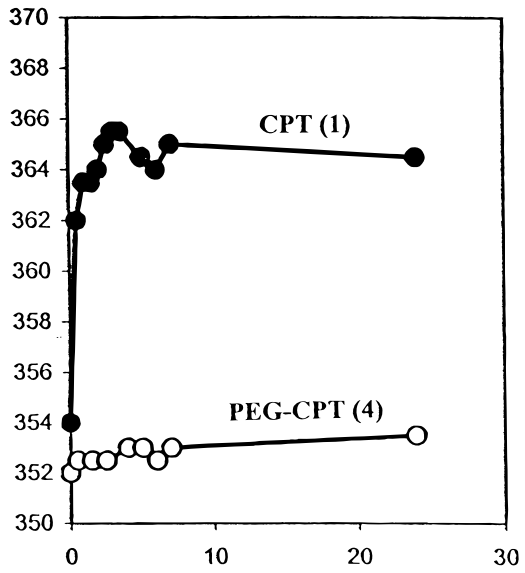
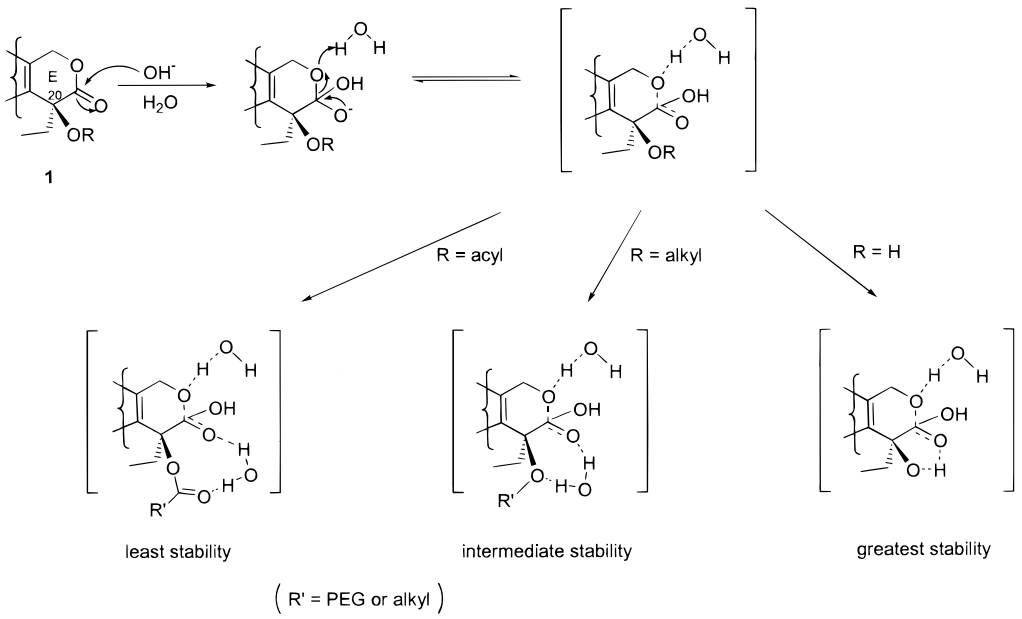


Figure 3. Kinetic profile (wavelength vs time) of CPT (**1**) and PEG-CPT (**4**) in pH 7.4 buffer at 37 °C for 24 h.

The NMR and UV studies clearly show that under physiological conditions 20-*O*-acyl-derivatives of CPT exist in the lactone form and show no indication of an equilibrium with an open carboxylate form. Hydrolytic studies further substantiate the results of the physical measurements.

From pH 7.4 to pH 9.5, no open forms for compounds **4**, **8**, **9**, or **10** could be detected, although some free CPT in the open carboxylate form was found which probably arose from the exocyclic hydrolytic release of CPT as discussed below. The nonpegylated small molecule, **10** (CPT-20-*O*-methyl carbonate), was synthesized and employed in the RP-HPLC studies as a model compound which could be easily analyzed, and which also eliminated any possibility of PEG effecting the outcome of the lactone equilibrium. This small CPT derivative essentially replicated the results obtained for **4**, **8**, and **9**. Although **10** released about 13% of native CPT at pH 9.5, this, again, most likely arose from the initial exocyclic cleavage of the carbonate since no open form of the methyl carbonate **10** could be detected in the basic media. Once released, native CPT quickly equilibrated to the more stable open carboxylate form. As previously men-

Scheme 5



tioned, studies have shown that the lactone ring opening of CPT is assisted by the 20-OH group.[13] When that 20-OH group is capped by an acyl derivative and no longer is capable of hydrogen bonding, opening of the E-ring becomes much more difficult. It appears from Table 2 that endocyclic ring opening at higher pH (> 9.5) cannot compete with the more rapid hydrolysis of the exocyclic acyl group, and more than 2 additional pH units is still insufficient for ring opening of these types of derivatives to occur.

We next explored the possibility of obtaining a carboxylate form of CPT with a nonacyl functionalized 20-OH group. For this purpose, compound **12**, a small molecule modified with a stable ether linkage at the 20-OH position was synthesized and studied under the same hydrolytic conditions as those employed for **10**. For the first time in this study an open form of a capped CPT derivative was detected by RP-HPLC and increased from 1.5% to 45% between pH 7.4 and 9.5. Nonetheless, compared to native CPT, the stability of the E-ring in compound **12** has greatly increased: only a negligible amount of open form CPT derivative formed at pH 8. When the pH was increased to 9.5, nearly half of **12** was converted to the open carboxylate form (**13**). Although the lactone ring in **10** and **12** is much more stable than in native CPT, there are substantial differences between these two compounds as well. At pH 9.5, 45% of **12** was found in the carboxylate form, whereas none of the open form was seen for **10**. The reason for this large difference in lactone ring stability probably arises from the different types of hybridization of the carbon atoms directly attached to the 20-OH. In compound **10**, the C-20 oxygen electron pair resonates with a sp^2 hybridized carbonyl thus preventing any substantial donor H-bonding. On the other hand for compound **12**, C-20 oxygen is attached directly to a sp^3 hybridized alkyl group and is capable of donor H-bonding with water. This latter situation is apparently not as effective for ring hydrolysis as having a free 20-OH which is capable of forming acceptor H-bonds as well: this type of H-bonding apparently creates a stable conformation very favorable to nucleophilic attack at the ring.[13] The various transition states of these 20-substituted CPT derivatives are depicted in Scheme 5. In the case of **12**, substantial hydrolysis of the E-ring is first observed at pH 9 where HO^- no doubt plays a role. Thus, by *not* engaging in hydrogen bonding in aqueous solution, an exocyclic acyl group at the 20-position results in a greatly stabilized lactone ring compared to a 20-exocyclic alkyl group. This feature might well find application in future CPT prodrug design regardless of the various mechanistic considerations that remain to be determined.

Conclusion

We have demonstrated by UV and NMR spectrophotometry, and HPLC analysis that acylation of the 20-OH group of CPT with PEG yields water soluble derivatives in which the lactone structure (E-ring) is stable under physiological conditions (pH 7.4, 37 °C). This is in marked contrast to native camptothecin where ring opening occurs very rapidly under the same circumstances. Our original hypothesis, inferred by lack of interaction of PEG-CPT (**3**) with HSA, that acylation of CPT stabilized the E-ring has thus been validated by the results of this physical and spectroscopic study. All CPT acyl derivatives prepared thus far appear to maintain the more bioactive lactone configuration and undergo only exocyclic cleavage at higher pH, generating native CPT, which rapidly undergoes ring opening under these conditions. This enhanced stabilization also precludes the possibility of the isolation of any PEG or alkyl open forms of acylated CPT by hydrolysis. To date, only in the case of an ether linkage at the 20-position at elevated pH (9.0) have we observed the open carboxylate form of CPT.

Experimental Section

Camptothecin was purchased from Boehringer Ingelheim KG. All other reagents and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI) and Advanced ChemTech (Louisville, KY). Unless stated otherwise, all reagents and solvents were used without further purification. All the PEGs used in this study had a molecular weight (MW) of 40000 and were dried under vacuum or by azeotropic distillation from

toluene prior to use. Organic solutions were dried over $MgSO_4$. Solvents were removed under reduced pressure. Deuterated chloroform was used as the solvent for ^1H and ^{13}C NMR unless otherwise specified. Analytical HPLC was conducted on a ZORBAX 300 SB C8 RP column (150 × 4.6 mm) with a gradient of 30 to 80% acetonitrile in 0.1 M triethylamine acetate (TEAA) buffer (pH = 6.8) at a flow rate of 1 mL/min. FAB-MS analyses were done at the Mass Spectrometry Facility, Comprehensive Cancer Center of Yale Medical School, New Haven, CT.

Synthesis. Preparation of PEG-20-*O*-Pro-CPT (4). A suspension of t-Boc-proline-N-hydroxyl succinimide ester (1.35 g, 4.31 mmol), scandium triflate[24] (1.00 g, 2.03 mmol), and 4-dimethyaminopyridine (DMAP, 1.24 g, 10.2 mmol) in anhydrous DCM (25 mL) was cooled in a ice-salt bath at 8 °C for 0.5 h. Camptothecin (0.50 g, 1.44 mmol) was then added and the reaction mixture stirred at −8 °C for 1 h, warmed to room temperature slowly and continuously stirred for 12 h. The reaction mixture was washed with 0.1 N HCl, distilled water and evaporated to dryness and dried under vacuum over P_2O_5 in desiccator for 24 h to give t-Boc-proline-20-*O*-CPT (0.72 g, 1.31 mmol) with a purity over 95% as checked by HPLC. This intermediate was then dissolved in a mixture of DCM (7 mL) and trifluoroacetic acid (3 mL) and stirred at room temperature for 0.5 h. The solvents were removed and redissolved in the minimum amount of methanol and precipitated with ethyl ether. Filtration gave TFA·Pro-20-*O*-CPT (0.55 g, 0.98 mmol) with a purity over 95% as checked by HPLC: ^1H NMR (270 MHz, $CDCl_3$ + CD_3OD) δ 8.50 (s, 1H), 8.21 (d, J = 8.1 Hz, 1H), 7.99 (d, J = 8.1 Hz, 1H), 7.83 (t, J = 8.1 Hz, 1H), 7.73 (s, 1H), 7.67 (t, J = 8 Hz, 1H), 7.29 (s, 1H), 5.30 (s, 2H), 4.90 (m, 2H), 4.08 (t, J = 8.1 Hz, 1H), 3.75 (m, 2H), 2.58 (s, 1H), 2.26 (m, 2H), 1.93 (m, 1H), 1.08 (t, J = 8.1 Hz, 3H); ^{13}C NMR (67.8 MHz, $CDCl_3$ + CD_3OD) δ 167.4, 165.3, 155.6, 150.8, 147.2, 145.2, 143.9, 130.2, 132.1, 127.8, 127.5, 127.1, 126.8, 117.5, 94.3, 76.5, 65.3, 57.3, 48.8, 44.1, 29.6, 27.2, 21.7, 6.3. This compound (0.16 g, 0.28 mmol) was then added to a solution of T-PEG[25] (2.5 g, 0.0625 mmol) and diisoproplylethylamine (DIEA, 0.54 mg, 0.416 mmol) in 10 mL of anhydrous DCM and stirred at room temperature for 12 h. Solvent was then removed and the residue recrystallized from 2-propanol (IPA) three times (50 mL, 65 °C) to give **4** (2.2 g, 86%): ^{13}C NMR (67.8 MHz) δ 170.72, 167.80, 167.00, 156.82, 153.00, 148.34, 145.75, 145.55,130.53, 129.85, 129.45, 128.05, 127.63, 127.58, 127.32, 118.81, 96.53, 77.19, 76.16−68.49 (PEG), 66.72, 57.81, 49.41, 45.62, 31.14, 29.11, 27.65, 24.31, 7.18.

Preparation of CPT-20-*O*-PEG Carbamate (8). A suspension of **1** (139 mg, 0.40 mmol), triphosgene (44 mg, 0.147 mmol), and DMAP (156 mg, 1.28 mmol) in anhydrous DCM (20 mL) was stirred for 10 min. PEG diamine[18] (2 g, 0.05 mmol) was added and the reaction mixture stirred for 16 h, evaporated to dryness and the residual solid recrystallized twice from IPA to give **8** (1.834 g, 90%): ^{13}C NMR (67.8 MHz) δ 172.22, 157.10, 154.43, 152.06, 148.67, 146.42, 144.33, 130.92, 130.83, 129.92, 129.84, 129.48, 127.65, 127.31, 120.00, 97.5, 72.28−67.58 (PEG), 66.57, 58.5, 50.00, 31.50, 7.10.

Preparation of CPT-20-*O*-PEG Carbonate (9). Prepared from PEG diol in 84% yield as described for **8**: ^{13}C NMR (67.8 MHz) δ 166.42, 156.87, 153.19, 152.14, 148.73, 146.15, 145.46, 130.70, 130.06, 129.45, 128.38, 127.97, 127.79, 127.52, 120.12, 95.29, 72.18−67.95 (PEG), 66.02, 61.33, 49.59, 31.61, 7.12.

Preparation of CPT-20-*O*-methyl Carbonate (10). A suspension of **1** (200 mg, 0.574 mmol), triphosgene (62 mg, 0.21 mmol), and DMAP (224 mg, 1.83 mmol) in anhydrous DCM (15 mL) was stirred for 10 min. Anhydrous methanol (15 mL) was added and the reaction mixture was stirred for 2 h, then evaporated to dryness in vacuo and the residue solid recrystallized from methanol/DCM to give **10** (198 mg, 85%): ^1H NMR (270 MHz) δ 8.41 (s, 1H), 8.24 (d, J = 8 Hz, 1H), 7.96 (d, J = 8 Hz, 1H), 7.85 (t, J = 8 Hz, 1H), 7.68 (t, J = 8 Hz, 1H), 7.34 (s, 1H), 5.56 (dd, J = 84 and 16 Hz, 2H), 5.30 (s, 2H), 3.79 (s, 3H), 2.23 (m, 2H), 1.01 (t, J = 8 Hz, 3H); ^{13}C NMR (67.8 MHz) δ: 167.06, 157.44, 154.34, 152.64, 149.25, 146.57, 145.94, 130.92, 130.58, 129.95, 128.65, 128.38, 128.13, 128.02, 120.77, 95.97, 77.96, 67.12, 55.32, 50.00, 31.19, 7.54; ESMS m/z 407 $(M + H)^+$.

Preparation of CPT-20-*O*-methylthiomethyl Ether (12). A mixture of **1** (0.50 g, 1.44 mmol), DMSO (30 mL), and acetic anhydride (10 mL) was stirred at room temperature for 2 days. The reaction solution was concentrated, and the residual solid diluted with ethyl acetate. The solution was washed with saturated aqueous $NaHCO_3$, brine, and water. The organic layer was dried over anhydrous $MgSO_4$, the solvent removed under reduced pressure, and the residue precipitated from methanol. The solid was collected by filtration and washed with hexane to give **12** (0.50 g, 83%): ^1H NMR (270 MHz) δ_8.41 (s, 1H), 8.25 (d, J = 8.1 Hz, 1H), 7.96 (d, J = 8.1 Hz, 1H), 7.85 (t, J = 8.1 Hz, 1H), 7.68 (t, J = 8.1 Hz, 1H), 7.28 (s, 1H), 5.61 (d, J = 16.2 Hz, 1H), 5.39 (d, J = 16.2 Hz, 1H), 5.32 (s, 2H), 4.57 (s, 2H), 2.28 (s, 3H), 2.10 (m, 2H), 0.92 (t, J = 8.1 Hz, 3H); ^{13}C NMR (67.80 MHz) δ 169.29, 157.46, 152.32, 148.93, 146.12, 145.26, 131.10, 130.65, 129.76, 128.33, 128.10, 128.04, 122.64, 97.55, 77.36, 71.03, 66.69, 49.93, 33.89, 14.90, 7.77; ESMS m/z 409 $(M + H)^+$.

General Methods. 1. NMR Studies. Compound **4** was treated as described below, and the recovered samples examined by NMR in order to determine if any equilibria had been established.

Method 1. Compound **4** (500 mg, 0.0125 mmol) was dissolved in 4 mL of PBS buffer (1 M, pH 7.4) and the solution incubated at 37 °C for 24 h. The buffered solution was then extracted with 20 mL of DCM twice, and the combined solutions dried over anhydrous magnesium sulfate and evaporated under reduced pressure to give a solid (480 mg).

Method 2. A solution of **4** (500 mg, 0.0125 mmol) in 16 mL PBS buffer (1 M, pH 7.4) was incubated at 37 °C for 24 h. The reaction mixture was lyophilized and the solid obtained suspended in DCM. The DCM layer was separated from insoluble solids in the suspension by centrifuging and decanting. The supernatant was evaporated and the residual solid (essentially quantitative recovery ∼500 mg) was dried under vacuum.

2. UV Studies. CPT samples were dissolved in a mixture of phosphate buffer and DMSO (10:1) and incubated in a water-bath at 37 °C. Aliquots were taken from the samples for scanning at time points of 0, 0.5, 1.5, 2.5, 4, 5, 6, 7, and 24 h.

3. Hydrolysis Studies. For PEG derivatives **4**, **8**, and **9**, 10 mg of each sample was dissolved in 1 mL of the appropriate phosphate buffer directly. The vials were then incubated at 37 °C for 24 h. Samples were analyzed using a HPLC. A modified procedure was used for the hydrolysis study of the water insoluble nonpegylated compounds **10** and **12**, 0.5 mg of the CPT derivative was dissolved in 1 mL phosphate buffer/DMSO (1:1) for different pH ranges.

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