Alkyl Esters of Camptothecin and 9-Nitrocamptothecin: Synthesis, in Vitro Pharmacokinetics, Toxicity, and Antitumor Activity

Zhisong Cao,* Nick Harris, Anthony Kozielski, Dana Vardeman, John S. Stehlin, and Beppino Giovanella*

The Stehlin Foundation for Cancer Research and St. Joseph Hospital Cancer Laboratory, 1918 Chenevert Street, Houston, Texas 77003

Received October 31, 1996[⊗]

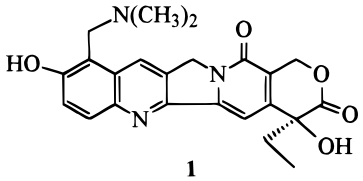
Eleven camptothecin esters, **6a**−**e** and **7a**−**f**, were prepared by straightforward acylation of camptothecins with the corresponding acylating reagents such as organic anhydrides and carboxylic acid chlorides. The in vitro pharmacokinetic determination of lactone levels of esters **6a** and **7b** showed that the biological life span of their lactone forms in human and mouse plasma significantly increased when compared with their mother compounds, camptothecin (**3**) and 9-nitrocamptothecin (**4**). The differences of lactone levels between human plasma and mouse plasma for **6a** and **7b** were much smaller than what was observed for their mother compounds. The in vivo antitumor activity and toxicity studies demonstrated that some of these esters were very active against human tumor xenografts in nude mice and had an exceptional lack of toxicity in nude mice, even at enormous doses.

Introduction

Camptothecin, a cytotoxic alkaloid first isolated from the wood and bark of *Camptotheca acuminata* (Nyssaceae) by Wall and co-workers, was shown to have antitumor activity against the mouse leukemia L1210 system.[1] The compound has a pentacyclic ring system with an asymmetrical center in ring E with 20*S* configuration. The pentacyclic ring system includes a pyrrolo[3,4-*b*]quinoline moiety (rings A, B, and C), a conjugated pyridone (ring D), and a six-membered lactone (ring E) with an α-hydroxyl group. Natural camptothecin itself is insoluble in water. Therefore, camptothecin was evaluated clinically as the water-soluble sodium carboxylate salt in the early stages. Unfortunately, this form of camptothecin produced severe toxicity and seemed devoid of anticancer activity,[2−6] which caused the discontinuation of phase II trials. However, the interest in camptothecin and its derivatives was revived by the finding that camptothecin inhibits topoisomerase I,[7−10] an enzyme that is required for swiveling and relaxation of DNA during molecular events such as replication and transcription.[11] A number of new syntheses and modifications of the molecule have been reported in the literature, and many new derivatives have been prepared over the years.[12−29]

In 1989, it was found that camptothecin and some of its semisynthetic derivatives possessed extraordinary anticancer activity against human cancer xenografts in nude mice.[30] Two groups of camptothecins have been developed leading to clinical trials since then. The first group, which can be called water-soluble camptothecins, consists of Topotecan (**1**)[20] and Irinotecan (**2**)[31] (Chart 1). Both of them are now commercially available as aqueous solutions which are injectable intravenously for human treatment. The second group, water-insoluble, comprises the mother compound, camptothecin (**3**), and its semisynthetic derivatives, 9-nitrocamptothecin (**4**), and 9-aminocamptothecin (**5**) (Chart 2). Many attempts have been made to modify the camptothecin structure in order to reduce its toxicity and maintain or increase its activity. While the derivatives made today have not met these criteria, they have offered important informa-

Chart 1. Topotecan (**1**) and Irinotecan (CPT-11, **2**)



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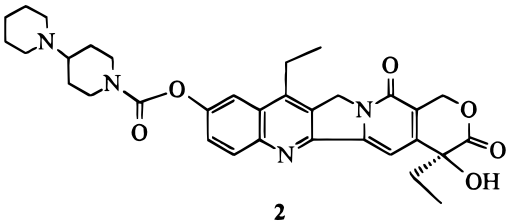

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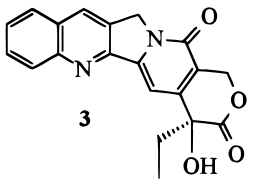
Chart 2. Camptothecin (**3**), 9-Nitrocamptothecin (**4**), and 9-Aminocamptothecin (**5**)



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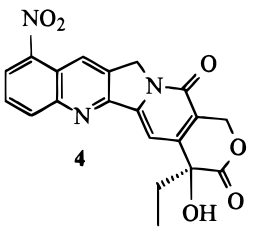

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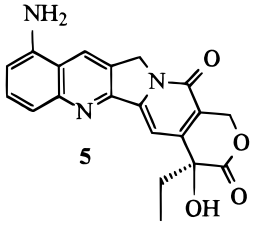

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[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1997.

S0022-2623(96)00756-X CCC: $15.00
Published on Web 01/01/1998

tion about critical structural features necessary for activity. For example, Wani and Wall demonstrated that the carboxylate sodium salt form of camptothecin has only about one-tenth the potency of **3** in one antitumor assay (P388 rodent leukemia),[16] implying that the intact lactone ring E of **3** is the most critical structural feature with respect to antitumor activity. It has also been shown that administration of **3** with closed lactone ring E is far superior to the injection of the water-soluble carboxylate salt in inhibiting growth of human cancer xenografts in nude mice.[32] This further confirmed the importance of the intact lactone ring E.

Extensive studies of the camptothecins used to treat human cancers growing as xenografts in nude mice showed very high activity against a wide spectrum of human neoplasms. In our laboratory,[33] we were able to obtain a response rate of 100% of all human cancers treated as established measurable tumors in nude mice (32/32). But, a response rate of only about 15% was observed from a recently concluded phase II trial of 9-nitrocamptothecin against ovarian cancer.[34] It is not surprising that results in mice are substantially better than in humans when comparing the plasma lactone levels of the drug in mice and humans. We found about 50% of the closed lactone form present in mice versus about 3% in humans when determining the percentage of the area under the curve (AUC) for 9-nitrocamptothecin (**4**). This means that the human tumor is exposed to a concentration of the active drug 15-fold higher in mice than in humans. It thus becomes evident that the crucial obstacle to improving camptothecin effectiveness is the rapid opening of the lactone ring in the human body.

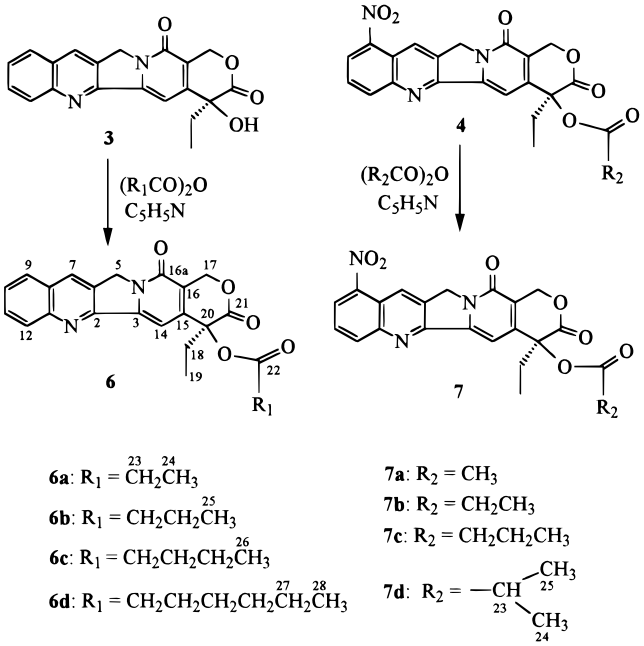
As seen above, prolonging the biological life of an active drug by protecting the lactone moiety of the molecule is important to drug development. An attempt[35] made by Wall et al. was to convert some synthetic (*RS*)-camptothecin derivatives into the corresponding water-soluble glycinate hydrochloric salts. These glycinate hydrochloric salts were tested in vivo against L1210 cells and showed less potency when compared with camptothecin. Whether these glycinates could prolong the biological life of the active drug was not addressed.

Thus, we propose the intact lactone ring would be better protected if camptothecins were transformed into the correponding water-insoluble alkyl esters. In this paper, synthesis and biological activity of camptothecin and 9-nitrocamptothecin esters, **6a**−**e** and **7a**−**f**, are described.

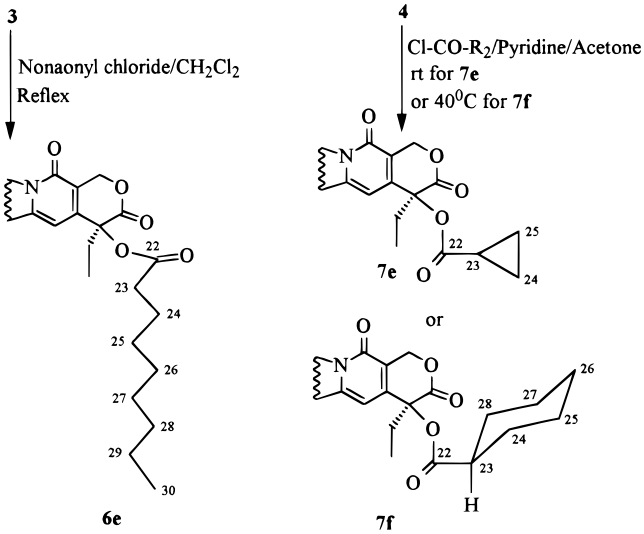
Chemistry

Camptothecin esters **6a**−**d** were prepared in high yields by the straightforward acylation of camptothecin with the corresponding organic anhydrides (Scheme 1). Thus, compound **6a** was obtained in 94% yield by the reaction of **3** with propionic anhydride in pyridine at 40 ± 5 °C. In the same manner, compounds **6b**−**d** were obtained in yields of 92%, 90%, and 98%, respectively, when the corresponding butyric anhydride, valeric anhydride, and heptanoic anhydride were used as the acylating reagents. The ^{1}H and ^{13}C NMR spectra of these esters showed the corresponding characteristic absorptions for their side ester alkyl chains.

Scheme 1



Scheme 2



Compounds **7a**−**d** were prepared in moderate to high yields by the esterification of 9-nitrocamptothecin (**4**) with the corresponding organic anhydrides (Scheme 1). Thus, product **7a** was obtained in 45% yield by the reaction of **4** with acetic anhydride. The acetyl methyl protons absorbed at 2.26 ppm. Product **7b** was obtained in 73% yield by reacting **4** with propionic anhydride. The ^{1}H NMR spectrum of **7b** showed a characteristic triplet with a *J* value of 7.46 Hz at 1.18 ppm for the C24-methyl group and a multiplet at 2.52−2.70 ppm for the C23-methylene protons. On reacting **4** with butyric anhydride or isobutyric anhydride, products **7c**,**d** were obtained in yields of 56% and 14%, respectively. The corresponding ^{1}H NMR peaks for the side propyl group of **7c** and the side isopropyl group of **7d** were observed.

The products **6e** and **7e**,**f** were prepared by using the corresponding carboxylic acid chlorides as the acylating reagents (Scheme 2). Product **6e** was obtained in 6% yield by acylation of **3** with nonanoyl chloride in methylene chloride. Its ^{1}H NMR spectrum obtained from

Table 1. Comparison of Percent Lactone of Prodrug **6a** and Camptothecin (CPT) in Human and Mouse Plasma

	time (h)				
	0	1	2	4	6
human plasma					
% lactone for **6a**	100.0	86.0	77.0	68.0	56.0
% lactone for CPT	100.0	12.0	0.50	00.0	00.0
mouse plasma					
% lactone for **6a**	100.0	97.5	ND	ND	85.0
% lactone for CPT	100.0	42.0	35.0	18.0	18.0

Table 2. Comparison of Percent Lactone of Prodrug **7b** and 9-Nitrocamptothecin (9NC) in Human and Mouse Plasma

	time (h)				
	0	1	6	28	51
human plasma					
% lactone for **7b**	100.0	93.7	64.4	16.6	5.8
% lactone for 9NC	100.0	7.0	00.0	00.0	00.0
mouse plasma					
% lactone for **7b**	100.0	97.7	84.3	73.1	59.9
% lactone for 9NC	100.0	30.0	18.0	18.0	ND

$CDCl_3$ showed a multiplet for those methylene protons in the long side ester alkyl chain. On reacting **4** with the corresponding cyclopropanecarboxylic acid chloride or cyclohexanecarboxylic acid chloride, products **7e**,**f** were obtained in yields of 30% and 63%, respectively. The corresponding characteristic proton peaks for the side cyclopropyl group of **7e** and the side cyclohexanyl group of **7f** were observed.

The mass spectral data of esters **6a**−**e** showed characteristic decomposition patterns. They all gave the M + H peaks and the peaks at *m/e* 331, 317, 303, and 287. The mass spectral data of esters **7a**−**f** also showed characteristic fragmentation patterns. They all gave the molecular ion peaks and peaks at *m/e* 375, 360, 347, 319, and 302.

Results and Discussion

The in vitro determination of lactone levels in human and mouse plasma for **6a** and **7b** are shown in Tables1 and 2. As shown in Table 1 the percent lactone of camptothecin (**3**) in human blood is 12% after 1 h, 0.5% after 2 h, and undetectable after 4 h. In other words, the active form of **3** is completely lost in a very short time period after oral administration. This is the opposite to what is observed in mice, in which the active drug form (i.e., the closed lactone form) lasts for a very long time period. For example, the closed lactone form of **3** in mouse plasma is still detectable after 6 h (18%, Table 1). The closed lactone form of **6a** in human plasma is much more stable than its mother compound **3**. For example, 56% of **6a** is still detected as the closed lactone form even after 6 h (Table 1). Camptothecin, in terms of lactone level, shows a big difference between mouse and human. This kind of difference is not observed for prodrug **6a**. Even more striking are the results obtained with prodrug **7b**. As shown in Table 2, after oral administration of this compound, the lactone form of **7b** is very stable in human and mouse plasma. While its mother compound **4** stays in human plasma as the closed lactone form for only a very short time period, the majority of the drug present in human plasma is the toxic carboxylate salt form. It is also evident that the differences of lactone levels in plasma between human and mouse for **7b** are much smaller than what is observed for its mother compound **4**.

Table 3. Changes in Body Weights of Mice during the Test Time Period

doses (mg/kg)	time (days)/body weight (g)					
control	21/31.7	27/33.5	34/34.4	41/35.2	48/35.0	56/36.7
5	21/33.0	27/33.7	34/34.4	41/33.5	48/32.9	56/33.2
6	21/32.9	27/33.4	34/33.8	41/33.5	48/34.0	56/32.2
7	21/30.8	27/31.7	34/30.6	41/31.1	48/31.6	56/31.5
8	21/32.9	27/34.1	34/34.0	41/33.4	48/33.9	56/33.2

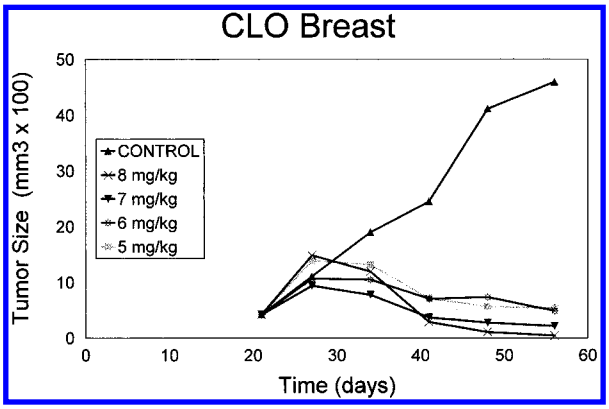


Figure 1. Activity of prodrug **6a** against human breast carcinoma.

All 11 esters were tested in vivo with nude Swiss mice of the NIH strain as the animal models. Esters **6a** and **7b** showed the best results. The in vivo toxicity study showed that these esters had little or no toxicity in nude mice. Overall toxicity can be judged using various criteria. For example, loss of body weight in a subject over 10% of the initially recorded body weight (i.e., before treatment) can be considered as one sign of toxicity. In addition, loss of mobility and activity and signs of diarrhea or cystitis in a subject can also be interpreted as evidence of toxicity. Table 3 shows the toxicity of prodrug **6a** in nude mice with different doses. The change of body weight of mice is recorded as a function of time. Body weight losses in mice during the test time period were not observed.

These ester compounds exhibit significant antitumor activity against human cancer xenografts growing in nude mice. Figure 1 represents the antitumor activity of prodrug **6a** in different doses against the implanted human breast carcinoma CLO in nude mice. Figure 2 shows the antitumor activity of **6a** at a dose of 100 mg/kg of body weight against human lung carcinoma SPA in nude mice. Figure 3 shows the anticancer activity of prodrug **7b** at a dose of 30 mg/kg of body weight against human colon carcinoma SQU in nude mice.

Conclusion

We have described the synthesis of 11 camptothecin esters. The compounds selected for the in vitro determination of lactone levels showed that their biological life span in human and mouse plasma was much longer than that of their parent compounds. The differences of lactone levels in plasma between human and mouse for these tested esters, such as **6a** and **7b**, were much smaller than that observed for their parent compounds.

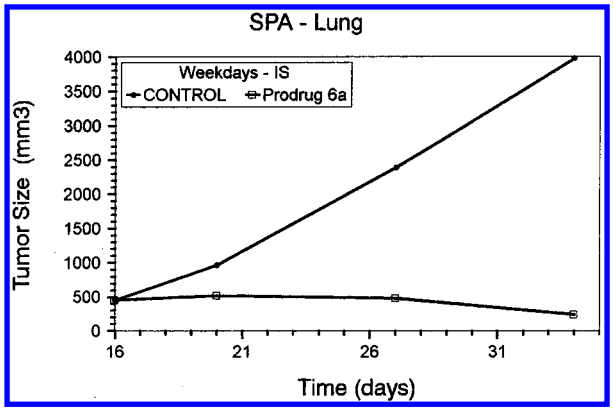


Figure 2. Activity of prodrug **6a** against human lung carcinoma.

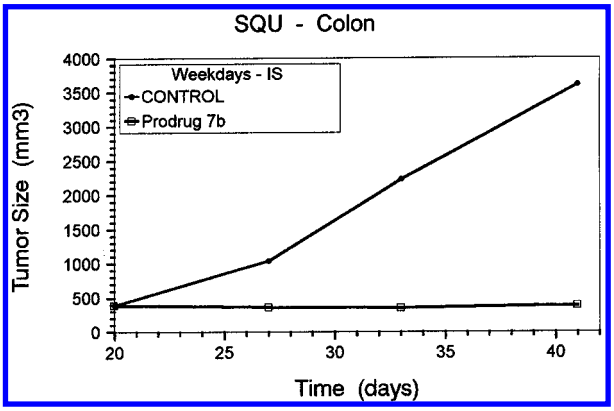


Figure 3. Activity of prodrug **7b** against human colon carcinoma.

The overall toxicity of these tested esters against nude mice when compared with their parent compounds was much lower. Meanwhile, their antitumor activity against xenografts of human tumors growing in nude mice is maintained.

The biological studies for these esters are very preliminary. Many other aspects such as topoisomerase I inhibition (IC_{50}), the cleavage of supercoiled DNA by topoisomerase I in the presence of these esters, and the rate of hydrolysis of these esters will be further studied.

Experimental Section

All glassware was baked at 80−100 °C for a minimum of 2 h before being used. Melting points were obtained with a MEL-TEMP melting point apparatus and are uncorrected. The ^{1}H and ^{13}C NMR spectra were obtained at 270.05 MHz with a JEOL GX-270 WB NMR spectrometer. Chemical shifts are reported in parts per million (δ scale), employing tetramethylsilane as an internal standard. In reporting the NMR data, we have used the following abbreviations: coupling constants in hertz (J), singlet (s), doublet (d), triplet (t), broad singlet (brs), multiplet (m), etc. Mass spectra were recorded using a VG ZAB-SEQ mass spectrometer (VG Analytical Co., England) with a resolution of 10 000. The numbering system used to depict NMR for these new camptothecin esters is shown in Schemes 1 and 2.

The starting camptothecin was purchased from The People's Republic of China and was purified before being used. The starting 9-nitrocamptothecin was prepared by following the procedure described by Wall et al.[17] The other organic anhydrides, carboxylic acid chlorides, and solvents used for the experiments were all purchased from Aldrich Chemical Co. (Milwaukee, WI).

Camptothecin-20-*O*-propionate (6a). In a 100-mL round-bottomed flask were mixed 25 mL of propionic acid anhydride and 20 mL of pyridine. A homogeneous solution was obtained after shaking for 30 s. To this solution was added 2.0 g of starting camptothecin. The mixture was stirred at 40 ± 5 °C for 48 h. After cooling to room temperature, the reaction mixture was poured onto 400 mL of petroleum ether while stirring. The product precipitated from petroleum ether was collected by filtration and washed with 150 mL of petroleum ether (50 mL × 3). After drying under air for 1 h, a white powder, 2.17 g, was obtained, yield 94%, purity 98% (HPLC), mp 250−252 °C dec. ^{1}H NMR in $CDCl_3$: δ 0.98 (3H, t, J = 7.5 Hz, C19-methyl protons), 1.17 (3H, t, J = 7.51 Hz, C24-methyl protons), 2.12−2.34 (2H, m, C18-methylene protons), 2.48−2.58 (2H, m, C23-methylene protons), 5.29 (2H, s, C5-methylene protons), 5.39−5.72 (2H, dd, J = 17.12, 17.12 Hz, C17-methylene protons), 7.23 (1H, s, C14-H), 7.68 (1H, t, J = 6.96 Hz, C10-H), 7.84 (1H, t, J = 6.96 Hz, C11-H), 7.95 (1H, d, J = 8.43 Hz, C9-H), 8.23 (1H, d, J = 8.06 Hz, C12-H), 8.40 (1H, s, C7-H). ^{1}H NMR in TFA: δ 1.18 (3H, t, J = 7.5 Hz, C19-methyl protons), 1.32 (3H, t, J = 7.30 Hz, C24-methyl protons), 2.30−2.80 (4H, m, C18- and C23-methylene groups), 5.60−6.10 (4H, s + dd, s at 5.86 for C5-methylene protons, dd with J = 18.96, 18.32 Hz for C17-methylene protons), 7.99 (1H, s, C14-H), 8.19 (1H, t, J = 8.06 Hz, C10-H), 8.20−8.46 (2H, m, C9-H, C11-H), 8.54 (1H, d, J = 8.79 Hz, C12-H), 9.43 (1H, s, C7-H). ^{13}C NMR (TFA): δ 7.42 (C19), 8.55 (C24), 28.61 (C18), 33.07 (C23), 53.14 (C5), 68.77 (C17), 78.31 (C20), 105.68, 113.19, 117.35, 125.87, 131.23, 131.36, 132.59, 133.40, 139.30, 139.89, 140.78, 144.62, 147.00, 149.43 (C2, C3, C6−C16, C16a), 172.50, 179.76 (C21, C22). MS *m/e* (relative intensity): 405 (M + H, 100), 404 (M^+, 15), 331 (M − CH_3CH_2COO, 17), 317 (M − C_2H_5COO − CH_3 + H, 10), 303 (M − C_2H_5COO − CO, 15), 287 (M − C_2H_5COO − CO_2, 9), 273 (8), 261(9). Precise mass ($C_{23}H_{20}N_2O_5$): found, 404.137; required, 404.137.

Camptothecin-20-*O*-butyrate (6b). Using 20 mL of butyric anhydride, 18 mL of pyridine, and 1.61 g of camptothecin, the reaction was carried out in the same manner as in preparing **6a**, yield 92%, purity 99% (HPLC), mp 225−227 °C dec. ^{1}H NMR in $CDCl_3$: δ 0.98 (6H, t, J = 7.51 Hz, C19- and C25-methyl groups), 1.65−1.74 (2H, m, C24-methylene protons), 2.14−2.30 (2H, m, C18-methylene protons), 2.44−2.51 (2H, m, C23-methylene protons), 5.29 (2H, s, C5-methylene protons), 5.38−5.71 (2H, dd, J = 17.59, 17.59 Hz, C17-methylene protons), 7.23 (1H, s, C14-H), 7.68 (1H, t, J = 8.06 Hz, C10-H), 7.84 (1H, t, J = 7.96 Hz, C11-H), 7.95 (1H, d, J = 6.96 Hz, C9-H), 8.23 (1H, d, J = 8.06 Hz, C12-H), 8.40 (1H, s, C7-H). ^{1}H NMR in TFA: δ 0.75−1.15 (6H, m, C19- and C25-methyl groups), 1.70−1.80 (2H, m, C24-methylene protons), 2.10−2.80 (4H, m, C18- and C23-methylene groups), 5.50−6.00 (4H, s + dd, s at 5.73 for C5-methylene protons, dd for C17-methylene protons), 7.86 (1H, s, C14-H), 8.05 (1H, s, C10-H), 8.30 (2H, brs, C9-H, C11-H), 8.40 (1H, s, C12-H), 9.30 (1H, s, C7-H). ^{13}C NMR (TFA): δ 7.23 (C19), 13.20 (C25), 19.20 (C24), 32.91 (C18), 36.91 (C23), 52.96 (C5), 68.58 (C17), 78.00 (C20), 105.56, 113.40, 113.50, 117.00, 117.10, 131.00, 132.40, 133.16, 139.06, 139.15, 140.00, 144.36, 146.90, 149.40 (C2, C3, C6−C16, C16a), 172.50, 178.00 (C12, C22). MS *m/e* (relative intensity): 419 (M + H, 100), 331 (M − C_3H_7COO, 17), 317 (M − $C_6H_{13}COO$ − CH_3 + H, 10), 303 (M − C_3H_7COO − CO, 13), 287 (M − C_3H_7COO − CO_2, 8), 273 (2), 261 (3). Precise mass ($C_{24}H_{22}N_2O_5$): found, 418.152; required, 418.153.

Camptothecin-20-*O*-valerate (6c). Using 15 mL of valeric anhydride, 14 mL of pyridine, and 1.51 g of starting camptothecin, product **6c** was obtained as a gray-white powder, yield 90%, purity 99% (HPLC), mp 265−267 °C dec. ^{1}H NMR in $CDCl_3$: δ 0.92 (3H, t, J = 7.33 Hz, C26-methyl protons), 0.98 (3H, t, J = 7.51, C19-methyl protons), 1.37−2.00 (4H, m, C24- and C25-methylene protons), 2.10−2.28 (2H, m, C18-methylene protons), 2.46−2.53 (2H, m, C23-methylene protons), 5.30 (2H, s, C5-methylene protons), 5.38−5.71 (2H, dd, J = 17.22, 17.21 Hz, C17-methylene protons), 7.23 (1H, s, C14-H), 7.70 (1H, t, J = 6.96 Hz, C10-H), 7.82 (1H, t, J = 6.96 Hz, C11-H), 7.95 (1H, d, J = 7.32 Hz, C9-H), 8.22 (1H, d, J = 8.42 Hz, C12-H), 8.40 (1H, s, C7-H). ^{1}H NMR in TFA: δ 0.83 (3H, brs, C26-methyl protons), 0.99 (3H, brs, C19-methyl

protons), 1.32 (2H, m, C25-methylene protons), 1.60 (2H, m, C24-methylene protons), 2.19−2.58 (4H, m, C18- and C23-methylene protons), 5.49−5.82 (4H, s + dd, s at 5.67 for C5-methylene protons, dd with J = 17.58, 18.68 Hz for C17-methylene protons), 7.80 (1H, s, C14-H), 7.99 (1H, s, C10-H), 8.23 (2H, brs, C9-H, C11-H), 8.33 (1H, s, C12-H), 9.24 (1H, s, C7-H). ^{13}C NMR (TFA): δ 4.48 (C26), 10.37 (C19), 20.23 (C24), 24.98 (C25), 30.15 (C18), 32.03 (C23), 50.20 (C5), 65.82 (C17), 75.36 (C20), 102.84, 109.89, 110.24, 114.06, 114.39, 128.25, 128.39, 129.65, 130.41, 136.30, 137.00, 141.62, 148.57, 149.28 (C2, C3, C6−C16, C16a), 169.00, 176.80 (C21, C22). MS *m/e* (relative intensity): 433 (M + H, 100), 331 (M − C_4H_9COO, 17), 317 (M − $C_6H_{13}COO$ − CH_3 + H, 10), 303 (M − C_4H_9COO − CO, 13), 287 (M − C_4H_9COO − CO_2, 7), 273 (2), 261 (4). Precise mass ($C_{25}H_{24}N_2O_5$): found, 432.168; required, 432.169.

Camptothecin-20-*O*-heptanoate (6d). Using 15 mL of heptanoic anhydride, 13 mL of pyridine, and 1.55 g of starting camptothecin, **6d** was obtained as a gray-white powder, yield 98%, purity 99% (HPLC), mp 270 °C (deformed at 210 °C). ^{1}H NMR in $CDCl_3$: δ 0.82 (3H, t, J = 7.51 Hz, C28-methyl protons), 0.98 (3H, t, J = 7.01 Hz, C19-methyl protons), 1.20−1.80 (8H, m, C24-, C25-, C26-, and C27-methylene protons), 2.10−2.30 (2H, m, C18-methylene protons), 2.40−2.60 (2H, m, C23-methylene protons), 5.29 (2H, s, C5-methylene protons), 5.38−5.72 (2H, dd, J = 17.69, 17.22 Hz, C17-methylene protons), 7.23 (1H, s, C14-H), 7.68 (1H, t, J = 7.30 Hz, C10-H), 7.84 (1H, t, J = 7.42 Hz, C11-H), 7.95 (1H, d, J = 8.06 Hz, C9-H), 8.22 (1H, d, J = 8.32 Hz, C12-H), 8.40 (1H, s, C7-H). ^{1}H NMR in TFA: δ 0.74 (3H, s, C28-methyl protons), 0.99 (3H, s, C19-methyl protons), 1.21 (6H, brs, C25-, C26-, and C27-methylene protons), 1.62 (2H, s, C24-methylene protons), 2.10−2.30 (4H, m, C18- and C23-methylene groups), 5.50−6.00 (4H, s + dd, s at 5.67 for C5-methylene protons, dd for C17-methylene protons), 7.80 (1H, s, C14-H), 7.99 (1H, s, C10-H), 8.23 (2H, s, C9-H, C11-H), 9.24 (1H, s, C7-H). ^{13}C NMR (TFA): δ 8.63 (C19), 14.99 (C28), 24.66 (C27), 27.14 (C26), 31.07 (C24), 33.68 (C25), 34.29 (C18), 36.45 (C23), 54.34 (C5), 69.98 (C17), 79.50 (C20), 106.97, 114.39, 118.55, 127.11, 132.41, 133.79, 134.55, 140.46, 141.11, 142.00, 145.79, 148.14, 150.62, 153.00 (C2, C3, C6−C16, C16a), 180.57, 193.10 (C21, C22). MS *m/e* (relative intensity): 461 (M + H, 100), 331 (M − $C_6H_{13}COO$, 20), 317 (M − $C_6H_{13}COO$ − CH_3 + H, 10), 303 (M − $C_6H_{13}COO$ − CO, 15), 287 (M − $C_6H_{13}COO$ − CO_2, 8), 273 (2), 261 (2). Presice mass ($C_{27}H_{28}N_2O_5$): found, 460.199; required, 460.120.

Camptothecin-20-*O*-nonanoate (6e). To 40 mL of methylene chloride in a 100-mL round-bottomed flask were added 5 mL of nonanoyl chloride and 2 g of starting camptothecin. The mixture was stirred under reflux for 48 h. After the solvent was evaporated by a rotary evaporator, the residue was chromatographically separated with chloroform−methanol as eluent. Product **6e** (169 mg) was obtained as a pale yellow powder, yield 6%, purity 99% (HPLC), mp 180 °C. ^{1}H NMR in $CDCl_3$: δ 0.84 (3H, t, J = 6.60 Hz, C30-methyl protons), 1.02 (3H, t, J = 7.69 Hz, C19-methyl protons), 1.20−1.80 (12H, m, C24−C29-methylene protons), 2.10−2.38 (2H, m, C18-methylene protons), 2.40−2.60 (2H, m, C23-methylene protons), 5.33 (2H, s, C5-methylene protons), 5.40−5.80 (2H, dd, J = 17. 22, 17.22 Hz, C17-methylene protons), 7.26 (1H, s, C14-H), 7.71 (1H, t, J = 8.06 Hz, C10-H), 7.88 (1H, t, J = 8.43 Hz, C11-H), 7.99 (1H, d, J = 7.33 Hz, C9-H), 8.26 (1H, d, J = 8.79 Hz, C12-H), 8.44 (1H, s, C7-H). ^{1}H NMR in TFA: δ 0.96 (3H, s, C30-methyl protons), 1.24 (3H, s, C19-methyl protons), 1.38 (10H, brs, C25−C29-methylene protons), 1.87 (2H, m, C24-methylene protons), 2.40−2.90 (4H, m, C18- and C23-methylene protons), 5.74−6.07 (4H, s + dd, s at 5.91 for C5-methylene protons, dd with J = 17.90, 18.21 Hz for C17-methylene protons), 8.05 (1H, s, C14-H), 8.24 (1H, t, C10-H), 8.48 (2H, m, C9-H, C11-H), 8.57 (1H, d, C12-H), 9.48 (1H, s, C7-H). ^{13}C NMR (TFA): δ 4.99 (C30), 11.45 (C19), 21.17 (C29), 23.53 (C28), 27.82 (C24, C26−C27), 30.52 (C25), 30.63 (C18), 32.80 (C23), 50.68 (C5), 66.34 (C17), 75.82 (C20), 103.28, 110.73, 114.91, 123.47, 128.79, 128.90, 130.14, 130.93, 136.84, 137.46, 138.33, 142.17, 144.47, 146.94 (C2, C3, C6−C16, C16a), 169.98, 176.92 (C21, C22). MS *m/e* (relative intensity): 489 (M + H, 100), 331 (M − $C_8H_{17}COO$, 23), 317 (M − $C_8H_{17}COO$ − CH_3 + H, 13), 303 (M − $C_8H_{17}COO$ − CO, 17), 287 (M − $C_8H_{17}COO$ − CO_2, 8), 273 (3), 216 (2). Precise mass ($C_{29}H_{32}N_2O_5$): found, 488.230; required, 488.231.

9-Nitrocamptothecin-20-*O*-acetate (7a). Acetic anhydride (3 mL) and pyridine (2 mL) were mixed in a 50-mL round-bottomed flask in which 140 mg of 9-nitrocamptothecin was placed. The mixture was stirred at room temperature for 24 h. The mixture was then poured onto 200 mL of petroleum ether while stirring. The crude product was precipitated and collected by filtration. After separation by column chromatography with chloroform−methanol as eluent, the pure product **7a** (70 mg) was obtained as a yellow powder by reprecipitation from petroleum ether, yield 45%, purity 99% (HPLC), mp 195 °C (deformed at 165 °C). ^{1}H NMR ($CDCl_3$): δ 1.02 (3H, t, J = 7.51 Hz, C19-methyl protons), 2.10−2.40 (5H, s + m, s at 2.26 for C23-methyl protons, m for C18-methylene protons), 5.40 (2H, s, C5-methylene protons), 5.41−5.75 (2H, dd, J = 17.59, 17.95 Hz, C17-methylene protons), 7.23 (1H, s, C14-H), 7.96 (1H, t, J = 6.96 Hz, C11-H), 8.53 (1H, d, J = 10.99 Hz, C10-H), 8.58 (1H, d, J = 9.98 Hz, C12-H), 9.31 (1H, s, C7-H). MS *m/e* (relative intensity): 435 (M^+, 25), 375 (M − CH_3COOH, 100), 360 (M − CH_3COOH − CH_3, 40), 347 (M − CH_3COOH − CO, 87), 332 (M − CH_3COOH − CO − CH_3, 37), 319 (13), 302 (11), 291 (10), 286 (11), 274 (10), 258 (4), 246 (5), 216 (8). Precise mass ($C_{22}H_{17}N_3O_7$): found, 435.107; required, 435.107.

9-Nitrocamptothecin-20-*O*-propionate (7b). Using 6 mL of propionic anhydride, 5 mL of pyridine, and 600 mg of starting 9-nitrocamptothecin, the pure compound (500 mg) was obtained as a yellow powder, yield 73%, purity 99% (HPLC), mp 163 °C (deformed at 155 °C). ^{1}H NMR ($CDCl_3$): δ 0.99 (3H, t, J = 7.51 Hz, C19-methyl protons), 1.18 (3H, t, J = 7.46 Hz, C24-methyl protons), 2.10−2.30 (2H, m, C18-methylene protons), 2.52−2.70 (2H, m, C23-methylene protons), 5.37 (2H, s, C5-methylene protons), 5.39−5.73 (2H, dd, J = 17.58, 17.58 Hz, C17-methylene protons), 7.22 (1H, s, C14-H), 7.93 (1H, t, J = 8.06 Hz, C11-H), 8.50 (1H, d, J = 10.60 Hz, C10-H), 8.54 (1H, d, J = 8.43 Hz, C12-H), 9.28 (1H, s, C7-H). MS *m/e* (relative intensity): 449 (M^+, 28), 375 (M − C_2H_5COOH, 100), 360 (M − C_2H_5COOH − CH_3, 35), 347 (M − C_2H_5COOH − CO, 82), 332 (M − C_2H_5COOH − CO − CH_3, 26), 319 (9), 302 (8), 291 (7), 274 (7), 258 (2), 245 (2), 216 (2). Precise mass ($C_{23}H_{19}N_3O_7$): found, 449.122; required, 449.122.

9-Nitrocamptothecin-20-*O*-butyrate (7c). Using 2 mL of butyric anhydride, 2 mL of pyridine, and 60 mg of starting 9-nitrocamptothecin, the product **7c** (40 mg) was obtained as a yellow powder, yield 56%, purity 99% (HPLC), mp 182 °C. ^{1}H NMR ($CDCl_3$): δ 0.98 (6H, m, C19- and C25-methyl groups), 1.65−1.70 (2H, m, C24-methylene protons), 2.10−2.40 (2H, m, C18-methylene protons), 2.41−2.60 (2H, m, C23-methylene protons), 5.36 (2H, s, C5-methylene protons), 5.38−5.72 (2H, dd, J = 17.59, 17.96 Hz, C17-methylene protons), 7.22 (1H, s, C14-H), 7.92 (1H, t, J = 7.52 Hz, C11-H), 8.49 (1H, d, J = 10.80 Hz, C10-H), 8.53 (1H, d, J = 9.53 Hz, C12-H), 9.27 (1H, s, C7-H). MS *m/e* (relative intensity): 463 (M^+, 14), 375 (M − C_3H_7COOH, 100), 360 (M − C_3H_7COOH − CH_3, 32), 347 (M − C_3H_7COOH − CO, 78), 332 (M − C_3H_7COOH − CO − CH_3, 25), 319 (9), 302 (8), 291 (7), 274 (7), 258 (2), 245 (3), 216 (5). Precise mass ($C_{24}H_{21}N_3O_7$): found, 463.137; required, 463.138.

9-Nitrocamptothecin-20-*O*-isobutyrate (7d). By the same manner as for the preparation of **7c**, product **7d** was obtained as a yellow powder, yield 14%, purity 99%, mp 197 °C. ^{1}H NMR ($CDCl_3$): δ 0.96 (3H, t, J = 7.52 Hz, C19-methyl protons), 1.21 (6H, t (d + d), J = 6.99, 6.99 Hz, C24- and C25-methyl groups), 2.00−2.30 (2H, m, C18-methylene protons), 2.60−2.80 (1H, m, C23-tertiary proton), 5.32 (2H, s, C5-methylene protons), 5.33−5.67 (2H, dd, J = 17.35, 17.35 Hz, C17-methylene protons), 7.20 (1H, s, C14-H), 7.88 (1H, dd, J = 8.08, 8.28 Hz, C11-H), 8.45 (1H, d, J = 7.51 Hz, C10-H), 8.50 (1H, d, J = 8.54 Hz, C12-H), 9.23 (1H, s, C7-H). ^{13}C NMR ($CDCl_3$): δ 7.60 (C19), 18.65 (C24, C25), 31.84 (C18), 33.60 (C23), 50.24 (C5), 67.00 (C17), 75.41 (C20), 96.80, 120.99,

121.54, 125.82, 127.46, 128.57, 131.53, 136.56, 145.00, 146.16, 148.91, 153.00, 157.23 (C2, C3, C6−C16, C16a), 167.23, 175.88 (C21, C22). MS *m/e* (relative intensity): 463 (M^+, 48), 375 (M − $(CH_3)_2CHCOOH$, 100), 360 (M − $(CH_3)_2CHCOOH$ − CH_3, 85), 347 (M − $(CH_3)_2CHCOOH$ − CO, 100), 332 (M − $(CH_3)_2$-CHCOOH − CO − CH_3, 72), 319 (23), 302 (24), 291 (17). Precise mass ($C_{24}H_{21}N_3O_7$): found, 463.138, required, 463.138.

9-Nitrocamptothecin-20-*O*-cyclopropanecarboxylate (7e). The starting 9-nitrocamptothecin (0.5 g, 0.0013 mol) and cyclopropanecarboxylic acid chloride (8 mL) were added to 20 mL of acetone in a 100-mL round-bottomed flask equipped with a magnetic stirrer. To this mixture was added 7 mL of pyridine dropwise while stirring. After stirring at room temperature for 15 h, the mixture was poured onto 750 mL of 5% hydrochloric acid solution in water while stirring. The yellow suspension obtained was extracted with 400 mL of methylene chloride (100 mL × 4). The combined extracts were washed with 200 mL of distilled water and dried over anhydrous sodium sulfate for 4 h. After filtration the solvent was removed by a rotary evaporator. The residue was chromatographically separated with methanol−chloroform as eluent. The pure product was obtained as a yellow powder, yield 30%, purity 99% (HPLC), mp 274 °C. ^{1}H NMR ($CDCl_3$): δ 0.62−1.07 (7H, m, C19-methyl, C24- and C25-methylene groups), 1.70−1.80 (1H, m, C23-tertiary proton), 2.10−2.70 (2H, m, C18-methylene protons), 5.32 (2H, s, C5-methylene protons), 5.33−5.65 (2H, dd, J = 17.35, 17.35 Hz, C17-methylene protons), 7.23 (1H, s, C14-H), 7.88 (1H, t (d + d), J = 8.03, 8.28 Hz, C11-H), 8.44 (1H, d, J = 7.51 Hz, C10-H), 8.51 (1H, d, J = 8.55 Hz, C12-H), 9.23 (1H, s, C7-H). ^{13}C NMR ($CDCl_3$): δ 7.60 (C19), 9.19, 9.36 (C24, C25), 12.78 (C23), 31.90 (C18), 50.20 (C5), 67.00 (C17), 75.64 (C20), 96.99, 120.99, 121.59, 125.81, 127.46, 128.61, 131.55, 136.51, 144.94, 146.04, 148.92, 153.86, 157.21 (C2, C3, C6−C16, C16a), 167.00, 173.75 (C21, C22). MS *m/e* (relative intensity): 461 (M^+, 13), 375 (M − cyclopropanecarboxylic acid, 100), 360 (M − cyclopropanecarboxylic acid − CH_3, 30), 347 (M − cyclopropanecarboxylic acid − CO, 66), 332 (M − cyclopropanecarboxylic acid − CO − CH_3, 27), 319 (9), 302 (8), 291 (4). Precise mass ($C_{24}H_{19}N_3O_7$): found, 461.123; required, 461.122.

9-Nitrocamptothecin-20-*O*-cyclohexanecarboxylate (7f). The starting 9-nitrocamptothecin (0.38 g, 0.0010 mol) and cyclohexanecarboxylic acid chloride were added to 25 mL of acetone in a 100-mL round-bottomed flask equipped with a magnetic stirrer. To this mixture was added 5 mL of pyridine dropwise. The mixture was stirred at 40 ± 5 °C for 15 h. After workup and separation same as for product **7e**, the product **7f** was obtained as a yellow powder, yield 63%, purity 99% (HPLC), mp 186 °C. ^{1}H NMR ($CDCl_3$): 0.80−1.15 (5H, m, C19-methyl, C26-methylene protons), 1.20−2.40 (10H, m, C18-, C24-, C25-, C27-, and C28-methylene groups), 2.42−2.60 (1H, m, C23-tertiary proton), 5.36 (2H, s, C5-methylene protons), 5.37−5.72 (2H, dd, J = 17.34, 17.34 Hz, C17-methylene protons), 7.23 (1H, s, C14-H), 7.92 (1H, t (d + d), J = 8.02, 8.28 Hz, C11-H), 8.48 (1H, d, J = 7.77 Hz, C10-H), 8.56 (1H, d, J = 8.54 Hz, C12-H), 9.28 (1H, s, C7-H). ^{13}C NMR ($CDCl_3$): 7.63 (C19), 25.23 (C25, C27), 26.65 (C24, C28), 28.58 (C26), 31.84 (C18), 42.49 (C23), 50.20 (C5), 66.98 (C17), 75.32 (C20), 96.87, 120.99, 121.54, 125.75, 127.42, 128.51, 131.54, 136.56, 144.96, 146.02, 146.23, 148.96, 153.83, 157.21 (C2, C3, C6−C16, C16a), 167.23, 174.77 (C21, C22). MS *m/e* (relative intensity): 503 (M^+, 20), 375 (M − cyclohexanecarboxylic acid, 100), 360 (M − cyclohexanecarboxylic acid − CH_3, 33), 347 (M − cyclohexanecarboxylic acid − CO, 90), 332 (M − cyclohexanecarboxylic acid − CO − CH_3, 26), 319 (10), 302 (10), 291 (4). Precise mass ($C_{27}H_{25}N_3O_7$): found, 503.170; required, 503.169.

In Vitro Determination of Lactone Levels in Human and Mouse Plasma for 3 and 6a. To 1 mL of plasma (human or mouse) was added 500 ng of drug in methanol. The mixture was incubated at 37 °C for the following time points: 0.5, 1, 2, 4, and 6 h; 100-μL aliquots were taken for analysis. The percent lactone of camptothecin was determined by the ratio of lactone to total drug at each time point. The total drug at each time point was consistent with a relative average deviation of 2%. The percent lactone of **6a** was determined by the ratio of drug measured at each time point to drug measured at starting time point (t = 0 h). A Waters C-8 Sep-Pak cartridge was preconditioned by vacuuming through 1 mL of methanol and then 1 mL of H_2O; 100 μL of plasma was then added and vacuumed through. The salt was removed by eluting with 2 mL of 20% methanol in water. The lactone was eluted with 1 mL of a solution of 50% methanol/H_2O/0.1% acetic acid. For total drug determination, 100 μL of plasma was added to 1000 μL of 0.35% perchloric acid. The mixture was vortexed for 10 s and left for 5 min at room temperature. The solution was then passed through a preconditioned C-8 Sep-Pak cartridge and washed with 2 mL of 20% methanol in water. The total drug was then eluted with 1 mL of 50% methanol/water/0.1% acetic acid. HPLC analysis: 500 μL of solution obtained above was injected through a 2-mL loop onto a C-8 Microsorb column and chromatographed with 30% acetonitrile/water/0.1% acetic acid as mobile phase. The drug was detected by a fluorescence detector at 347/418 nm. The peak area was integrated, and the lactone levels of drug at different time points were determined.

In Vitro Determination of Lactone Levels in Human and Mouse Plasma for 4 and 7b. To 1 mL of plasma (human or mouse) was added 500 ng of drug in methanol. The mixture was incubated at 37 °C of the following time points: 0.5, 1, 2, 4, 6, 28, and 51 h. The percent lactone of **4** was determined by the ratio of lactone to total drug. The total drug at each time point was consistent with a relative average deviation of 2%. The percent lactone of **7b** was determined by the ratio of drug measured at each time point to drug measured at starting time point (t = 0 h). A Waters C-8 Sep-Pak cartridge was preconditioned by vacuuming through 1 mL of methanol and then 1 mL of H_2O; 100 μL of plasma was then added and vacuumed through. The salt was removed by eluting with 2 mL of 20% methanol in water. The lactone was eluted with 1 mL of a solution of acetonitrile, methanol, and 10 mM ammonium formate (1:4:5, pH 2.0) and collected. To this 1 mL of eluent was added 200 μL of Fe/H_2O (100 mg of reduced pentacarbonyliron/1 mL of H_2O). The mixture was vortexed for 10 s, sonicated for 30 min, and centrifuged at 6000*g* for 30 s. HPLC analysis: 500 μL of solution was taken from the top homogeneous layer of the above-centrifuged mixture, injected through a 2-mL loop onto a C-8 Microsorb column, and chromatographed with 45% methanol in water−10 mM ammonium formate (pH 2.0) as mobile phase. The drug was detected by a fluorescence detector at 360/455 nm. The peak area was integrated, and the lactone levels of drug at different time points were determined.

General Procedure for Measurements of in Vivo Toxicity and Antitumor Activity. All the animal experiments were performed on nude Swiss mice of the NIH high-fertility strain. They were bred and raised in our laboratory under strict pathogen-free conditions.[36] The tumors used for the anticancer activity determination were human xenografts originally obtained from human biopsies and then carried in our laboratory by serial transplantation from nude mouse to nude mouse.[33] For the in vivo toxicity and antitumor activity determination, a tumor xenograft growing in a nude mouse, approximately 1 cm^3 in size, was excised sterilely, minced finely with iridectomy scissors, and suspended in MEM tissue culture medium at the ratio 1:10 (v/v). One-half of 1 mL of this suspension, containing about 50 mg of tumor mince wet weight, was inoculated subcutaneously on the upper half of the dorsal thorax of the mouse. Groups of six animals were used. The drug (ester) was finely suspended in cottonseed oil and then injected into the stomach cavity of a mouse through the anterior abdominal wall by using a 26-gauge needle. The weekly schedule previously established for 9-nitrocamptothecin injection was once a day, 5 days on, and 2 days off. This schedule was employed throughout all the animal experiments. Treatment was initiated when the tumor had reached a volume of about 200 mm^3, i.e., well-vascularized, measurable, and growing exponentially. Tumors growing in animals were checked and measured with a caliper once a week.

Acknowledgment. The authors wish to thank Mr. Edward Ezell at the University of Texas Medical Branch for NMR data and Baylor Medical College for mass data. Supporting funds from the Stehlin Foundation for Cancer Research and The Friends of The Stehlin Foundation are gratefully acknowledged.

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JM9607562