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Phosphate ester derivatives of homocamptothecin: Synthesis, solution stabilities and antitumor activities

Zhenyuan Miao [a,†], Jing Zhang [a,†], Liang You [b,†], Juan Wang [c], Chunquan Sheng [a,*], Jiangzhong Yao [a], Wannian Zhang [a,*], Hao Feng [a], Wei Guo [a], Lei Zhou [a], Wenfeng Liu [a], Linjian Zhu [a], Pengfei Cheng [a], Xiaoying Che [a], Wenya Wang [a], Chuan Luo [a], Yulan Xu [a], Guoqiang Dong [a]

[a] School of Pharmacy, Second Military Medical University, 325 Guohe Road, Shanghai 200433, People's Republic of China
[b] Department of Pharmacy, Naval general hospital of PLA, 6 Fucheng Road, Beijing 100037, People's Republic of China
[c] Department of Chemistry, Fudan University, 220 Handan Road, Shanghai 200433, People's Republic of China

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ABSTRACT

Homocamptothecins (hCPTs) represents a new promising class of topoisomerase I inhibitors with enhanced stability and superior antitumor activity. Some phosphodiesters and phosphotriesters homocamptothecin derivatives were designed and synthesized based on our previous synthetic route. The cytotoxicity in vitro on three cancer cell lines and antitumor activity in vivo, and inhibitory properties of topoisomerase I of these derivatives were evaluated. Among them compounds **24e** and **24f** exhibited higher cytotoxic activity than IRT and the former exhibited the best antitumor activity in vivo and solution stability both at pH 7.4 and pH 3.0.

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1. Introduction

20(*S*)-Camptothecin(CPT, **1**, Fig. 1), is a naturally occurring quinoline alkaloid from *Camptotheca acuminata* exhibiting an excellent antineoplastic activity against a broad spectrum of tumors.[1,2] The molecular target of CPT is DNA topoisomerase I (Topo I), a nuclear enzyme that catalyzes the relaxation of supercoiled DNA during DNA replication. It was found that CPT does not bind to Topo I or to DNA alone but forms a three-component complex by binding to a covalent DNA–Topo I complex, and thus inhibits DNA relaxation.[3–5] The structure–activity relationship (SAR) studies on CPT derivatives led to the approval of topotecan (Hycamtin, **2**) and irinotecan (Camptosar, **3**) for ovarian and colon cancers treatment, respectively, and to the synthesis of several novel CPT derivatives that are currently in various stages of clinical trials.[6–11]

Recent studies have shown that expansion of the camptothecin E-ring to a seven- membered system enhances the solution stability of the agent and exhibits better biological properties compared to CPT.[12] This new family of very active seven-member hydroxy lactone derivatives represent a new promising class of topoisomerase I inhibitors. Among these homocamptothecin(hCPT, **4**) derivatives, 9,10-difluoro analog (diflomotecan, **5**) is one of the most potent topoisomerase I inhibitors and was the first homocamptothecin to be selected for clinical trials.[13]

Our previous work has reported a new synthetic method to form 7-substituted homocamptothecin analogs by couples ring A with ring C, D, and E. These hCPT compounds exhibited potent antitumor activity in vitro superior to topotecan.[14] But for most compounds, their in vivo activity does not correlate well with their in vitro data because of their poor water solubility. In order to obtain the homocamptothecin derivatives with the excellent in vivo activity we designed and synthesized phosphate derivatives of 7-methyl-10-substituted homocamptothecin and explored whether this modification improves the biological properties and solution stability.

2. Results and discussion

2.1. Chemistry

The target compounds could be disconnected to ring A and ring CDE based on our reported synthetic method and preparation of these compound are illustrated in Schemes 1–3.The key intermediate CDE ring **19** was synthesized according to the reported route[14] and the synthetic process was optimized in our previous studies. First, six-member hydroxy lactone **15** was obtained from acetone and ethyl oxalate by 10-step reactions according to our reported synthetic route.[15–17] Then compound **15** was converted to tricyclic

* Corresponding authors. Tel./fax: +86 21 81871243 (C.S.).
E-mail addresses: shengcq@hotmail.com (C. Sheng), zhangwnk@hotmail.com (W. Zhang).
† These three authors contributed equally to this work.

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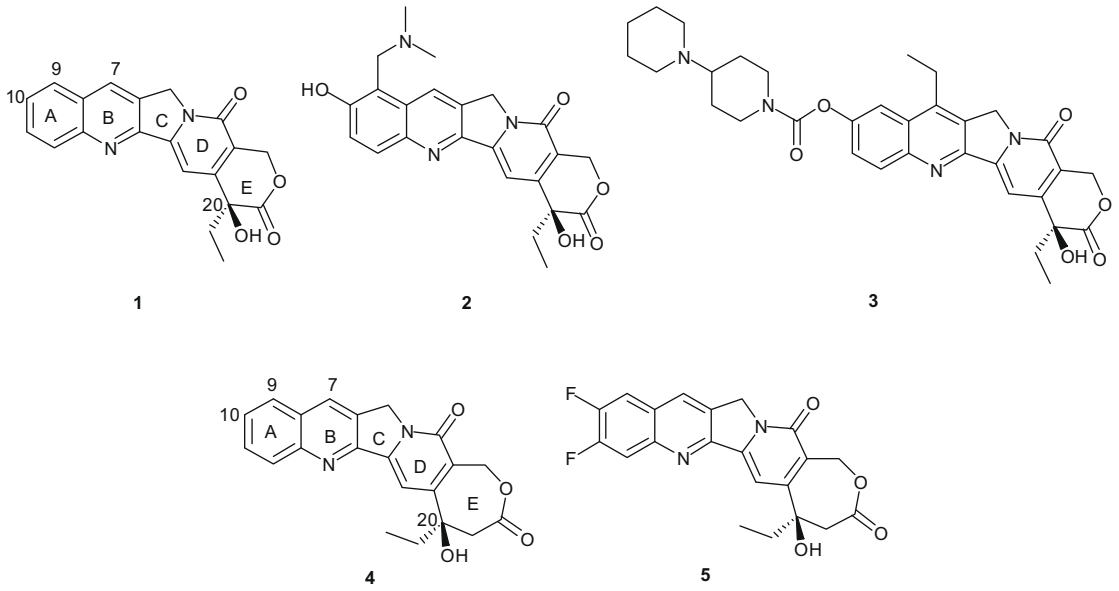
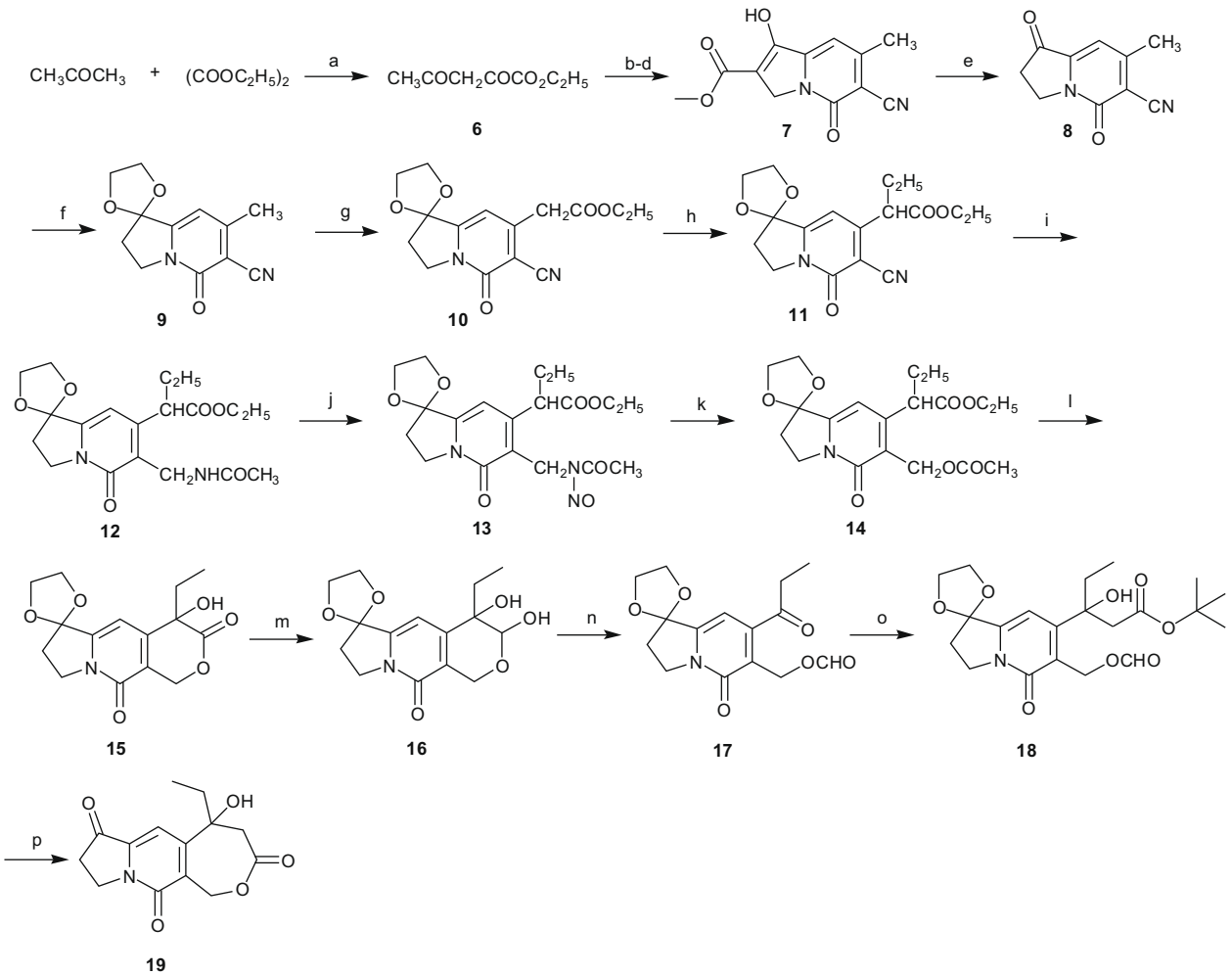
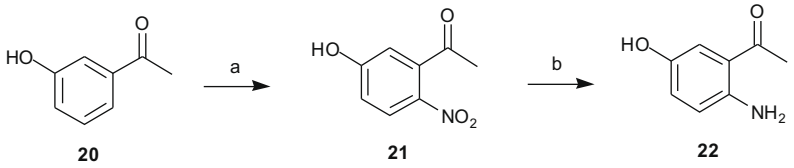


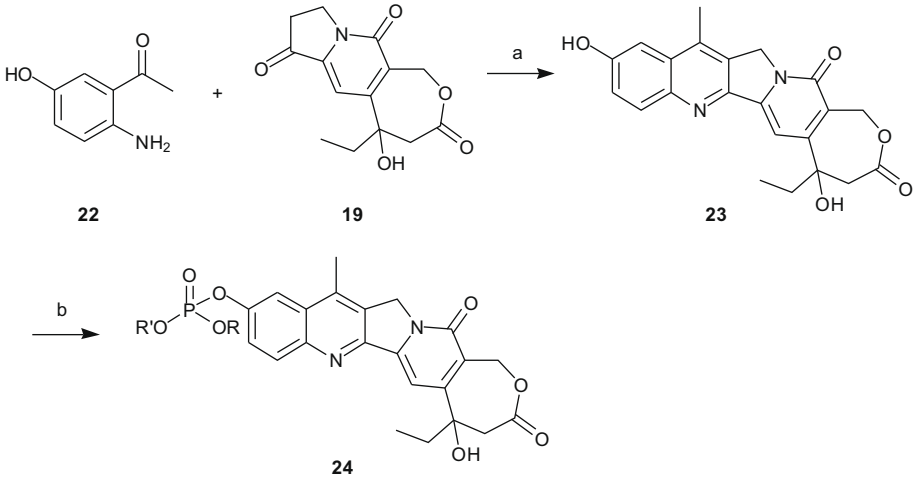
Figure 1. Structure of camptothecin and homocamptothecin derivatives.



Scheme 1. Reagents and conditions: (a)Na/CH_3OH, rt, 5 h; (b) triethyl orthoformate, *p*-TSA, 50 °C, 70 min; (c) cyanoacetamide, K_2CO_3, DMF, 60 °C, 16 h; (d) methyl acrylate, K_2CO_3, DMF, 55 °C, 72 h; (e) HCl, CH_3COOH, refluxing, N_2, 3 h; (f) $OHCH_2CH_2OH$, TMSCl, rt, 72 h; (g) diethyl carbonate, *t*-BuOK, DMF, refluxing, N_2, 3 h; (h)CH_2CH_3I, *t*-BuOK, DMF/TMF, −10 °C, 3 h; (i) Raney Ni, H_2, Ac_2O/HOAc, 50 °C, 12 h; (j) $NaNO_2$, 0 °C, 12 h; (k) CCl_4, refluxing, 10 h; (l) Raney Ni, CuCl, rt, 4 h, then H_2SO_4, rt, 0.5 h; (m) KBH_4, CH_3OH, rt, 20 min; (n) CH_3COOH, $NaIO_4$, rt, 0.5 h; (o) Zn, $BrCH_2COO^tBu$, THF, 75 °C, 3 h; (p) CF_3COOH, rt, 10 h.



Scheme 2. Reagents and conditions: (a) KNO_3, HOAc, 60 °C, 12 h; (b) Pd/C, H_2, 35 °C, 8 h.



24a: R=H,R'=Et
24b: R=H,R'=$C_6H_5CH_2$
24c: R=Et,R'=Et
24d: R=iso-Pr,R'=iso-Pr
24e: R=Bu,R'=Bu
24f: R=pentyl,R'=pentyl
24g: R=cetyl,R'=cetyl
24h: R=CF_3CH_2,R'=CF_3CH_2

Scheme 3. Reagents and conditions: (a) toluene, *p*-TSA, 120 °C, 4 h; (b) $POCl_3$, pyridine, Et_3N, a series of alcohols, 0 °C, 1.5 h.

intermediate **19** by reduction, oxidation, Reformatsky and lactonization reaction with a 67.1% yield (four steps).

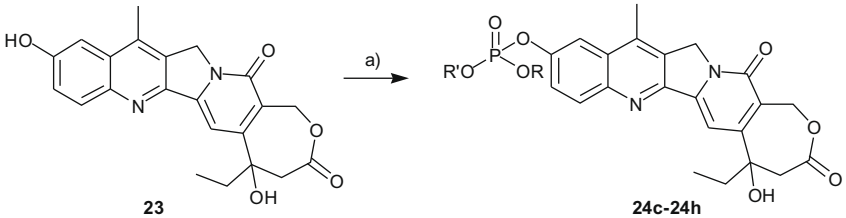
Then, in order to avoid using poisonous phosgene, acetic acid and KNO_3 were chosen for nitration reagents. Under this condition, 5-hydroxy-2-nitrohyacetophenone **21** was synthesized with a 33.7% isolated yield (Scheme 2). Hydrogenation of **21** in the presence of Pd/C afforded the amine **22** (Ring A).[18]

Finally, Friedlander condensation of the tricyclic **19** with **22** in the presence of *p*-toluenesulfonic acid gave 7-methy-10-hydroxy-homocamptothecin **23**, which was reacted with phosphoryl chloride and different alcohols in pyridine to give the homocamptothecin phosphodiesters **24a, 24b**, and phosphotriesters **24c–h** purifying through silica gel column chromatography. These reactions are shown in Scheme 3.

Furthermore, another synthesis method was applied to form homocamptothecin phosphotriesters by treatment with phosphorous trialkyl esters and *N*,*N*-dimethylamino-pyridine (DMAP) (Scheme 4). The reaction efficiently yielded the phosphorylated compounds **24c–h** in excellent yield (Fig. 2). Especially, when phosphorous tripentyl ester was used as phosphorylation agent, the yield remarkably increased from 12.2% to 96.0%.

2.2. In vitro antitumor activities

All the homocamptothecin analogs were assayed for cytotoxicity against three different human tumor cell lines (A549, MDA-MB-435, and LOVO) based on MTT assay. The data in Table 1 show that the antitumor activity of all homocamptothecin phosphates were much reduced. These phosphates were moderate cytotoxic to all three cell lines except compound **24g**. Among them, compounds **24e** and **24f** exhibited higher cytotoxic activity against three cancer cell lines than IRT. There is a decrease in potency against cancer cells for compound **24a** and **24b** indicating that the cytotoxicity of the phosphodiesters was lower than that of the phosphotriesters.



Scheme 4. Reagents and conditions: (a) I_2, phosphorous trialkyl esters, DMAP, CH_2Cl_2, 0–25 °C, 1.5 h.

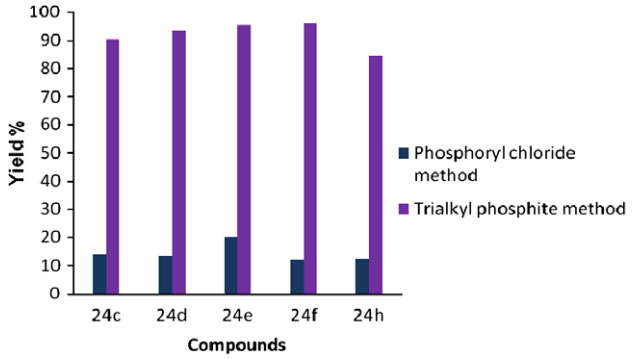


Figure 2. Comparison of two synthetic methods of homocamptothecin phosphotriesters.

Table 1
The in vitro antitumor activity of the target compounds

Compound	IC_{50} (μM)		
	A549	MDA-MB-435	LOVO
23	0.03	<0.001	0.01
24a	11.40	5.26	7.24
24b	29.35	10.77	9.31
24c	8.05	0.79	1.26
24d	11.39	3.40	5.56
24e	2.84	0.51	0.91
24f	2.30	0.72	1.19
24g	>100	>100	>100
24h	15.51	0.22	5.42
Topotecan	0.04	<0.001	0.02
Irinotecan	4.61	1.14	4.99

The SAR analysis of the synthesized compounds revealed that the alkyl-chain length of phosphates was important to in vitro cytoactivity. For example, the antitumor activity of compound **24e** with the butyl chain was far more than that of compound **24g** with the hexadecyl chain.

2.3. In vivo antitumor activities

Compound **24e** and compound **24f** were selected for evaluation in 5 week tumor growth inhibition in vivo assays using nude mice implanted with Lewis lung xenografts (Fig. 3). Irinotecan, a clinically relevant camptothecin, was chosen as a reference drug. Treatment with irinotecan at 50 mg/kg body weight, however, suppressed tumor growth by 51% as compared with placebo-treated controls. For compounds **24e** and **24f**, tumor growth was suppressed by 59% (25 mg/kg group) and 42% (50 mg/kg group), respectively. In addition, tumor regression was seen in a dose dependent manner for compound **24e** and compound **24f**. For example, the tumor growth inhibition rate of compound **24e** increased from 25% to 59% when the dose was increased from 6.25 mg/kg to 25 mg/kg.

2.4. Topoisomerase I-mediated DNA cleavage

The ability of selected homocamptothecin derivatives **23** and **24e** to inhibit topoisomerase I was investigated in the cleavable complex assay. The results indicated that compounds **23** as cytotoxic agents were higher potent than CPT (Fig. 4). For example, inhibit activity was observed for compound **23** at concentration of 1 μM while CPT showed no inhibit activity at the same concentration. Furthermore, the increasing concentration of **23** was accompanied by a dose-dependent increase in the level of cleavable complex. Compound **24e** also showed potent activity in the DNA cleavage assay at concentration of 100 μM.

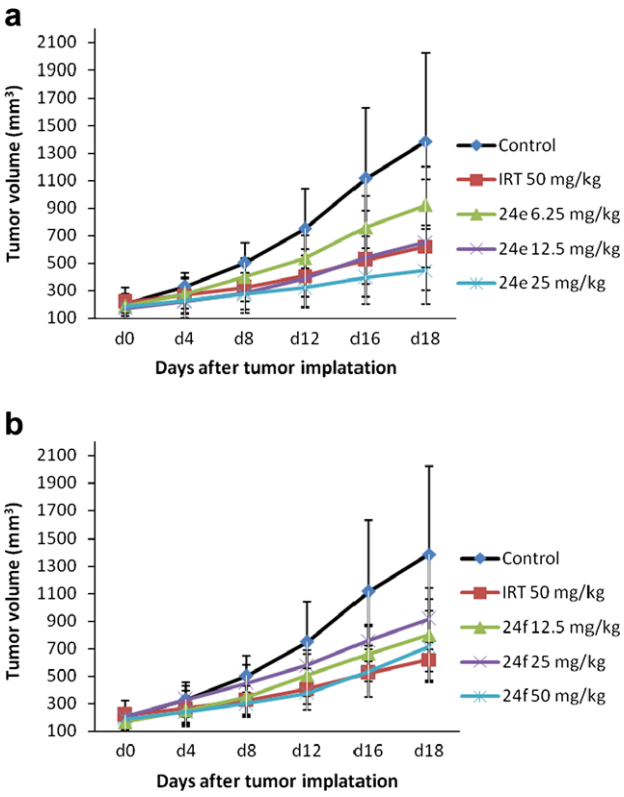


Figure 3. Tumor growth and inhibition of compounds **24e** (a) and **24f** (b) against A549 xenografts in nude mice administered ip.

2.5. Solution stability

The previously reported lactone stability study in buffer solutions showed the homocamptothecin to be considerably more stable than natural camptothecin product. In this article the phosphotriester stability of compounds **24e** and **24f** were determined by modified HPLC method.[19] The results showed that homocamptothecin phosphotriester derivatives were considerably stable both at pH 3.0 and pH 7.4. For example, after 24 h at pH 3.0, 100% of compounds **24e** and **24f** remained in phosphate form (some data not shown). Furthermore, after 24 h at pH 7.4, more than 95% of **24e** also remained in the phosphate form while only 91% of compound **24f** remained (Fig. 5).

3. Conclusion

In summary, the data presented here demonstrate that most phosphotriester derivatives possess potent cytotoxic activity in vitro and compound **24e** has more tumor inhibitory activity than IRT in the A549 xenograft model. The homocamptothecin phosphotriester derivative **24e** and **24f** as potent topoisomerase I inhibitors are stable not only at pH 7.4 but also at pH 3.0 as well. These results make phosphates a new class of homocamptothecin derivatives worthy of further investigation.

4. Experimental

4.1. General

Melting points were measured on an electrically heated XT4A instrument and are uncorrected. The ^1H spectra were recorded at 500 MHz with a Bruker instrument, and reported with TMS as internal standard and $CDCl_3$ or DMSO-d_6 as solvents. Chemical shifts (δ values) and coupling constants (J values) are given in

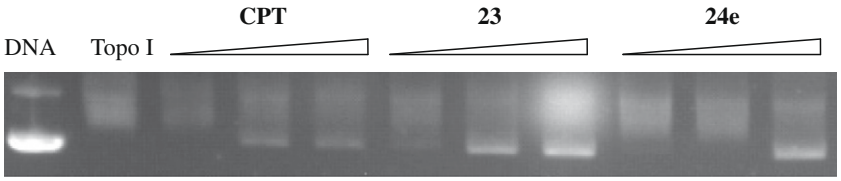


Figure 4. Effect of compounds **23** and **24e** on Topoisomerase I-mediated DNA relaxation. Lane DNA, supercoiled DNA pBR322; Lane Topo I, supercoiled DNA and Topo I. The samples were reacted with 1, 10, and 50 μM drug at 37 °C for 15 min.

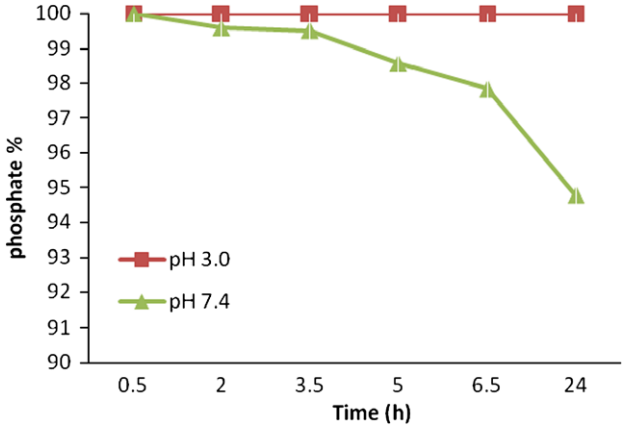


Figure 5. Solution stability of compounds **24e** at 37 °C. Each data point represents the percent of initial concentration of phosphate at pH 3.0 and pH 7.4, respectively.

ppm and Hz, respectively. ESI mass spectra were performed on an API-3000 LC–MS spectrometer. TLC analysis was carried out on silica gel plates GF254 (Qindao Haiyang Chemical, China). Flash column chromatography was carried out on silica gel 300–400 mesh. Anhydrous solvent and reagents were all analytical pure and dried through routine protocols.

4.2. Preparation of 1-(2-amino-5-hydroxyphenyl)ethanone (22)

4.2.1. Preparation of 1-(5-hydroxy-2-nitrophenyl)ethanone (21)

KNO_3 (11.1 g, 0.1 mol) was added to a solution of 3-hydroxyphenylethanone **20** (13.6 g, 0.1 mol) and acetic acid (100 mL) at ambient temperature. The mixture was stirred at 60 °C for 12 h and poured into ice-water. Then the solution was extracted with dichloromethane (3 × 100 mL). The combined dichloromethane extracts were dried over sodium sulfate and concentrated under reduced pressure giving crude material. Recrystallisation from ethyl acetate gave pale yellow needles **3** (6.1 g, 33.7%), mp 146–147 °C.

4.2.2. Preparation of 1-(2-amino-5-hydroxyphenyl)ethanone (22)

A suspension of compound **21** (1 g, 5.5 mmol) and Pd/C (0.1 g) in ethanol (30 mL) was degassed and refilled with N_2 for three times. Then the mixture was stirred under 20 psi of H_2 at 35 °C for 8 h. The catalyst was filtered and the solvent was removed by evaporation and yellow powder **22** (0.7 g, 83.9%) was obtained used directly in the next stage, mp 162–165 °C. ^{1}H NMR (500 MHz, DMSO-d_6, δ_{ppm}): 2.50 (s, 3H), 6.12 (s, 2H), 6.62 (d, 1H, J = 8.8 Hz), 6.82 (d, 1H, J = 8.8 Hz), 7.07 (s, 1H), 8.66 (s, 1H); ESI-MS, m/z: 152.1 $(M+H)^+$.

4.3. Preparation of 7-methy-10-hydroxyhomocamptothecin (23)

A solution of 1-(2-amino-5-hydroxyphenyl)ethanone **22** (0.5 g, 3.3 mmol) and 9-ethyl-9-hydroxy-2,3,8,9-tetrahydro-5*H*-6-oxa-3a-aza-cyclohepta[f]indene-1,4,7-trione **19**[14] (0.5 g, 1.8 mmol) in toluene (500 mL) was refluxed using a Dean–Stark trap for 30 min. *p*-Toluenesulfonic acid (0.1 g) was then added and refluxing was continued for an additional 4 h. The solution was allowed to cool to room temperature and the solid was filtered off and washed by acetone (20 mL) and methanol (20 mL) to give 7-methy-10-hydroxyhomocamptothecin **23** as yellow solid (0.5 g, 70.9%). mp >300 °C; ^{1}H NMR (500 MHz, DMSO-d_6, δ_{ppm}): 0.87 (t, 3H, J = 7.4 Hz), 1.86 (q, 2H, J = 7.4 Hz), 2.76 (s, 3H), 3.06–3.49 (q, 2H, J = 13.7 Hz), 5.28 (s, 2H), 5.39–5.53 (q, 2H, J = 15.1 Hz), 6.03 (s, 1H), 7.42 (s, 1H), 7.72 (d, 1H, J = 6.6 Hz), 7.87 (s, 1H), 8.15 (d, 1H, J = 9.2 Hz), 10.32 (s, 1H); ^{13}C NMR (DMSO-d_6): 8.60 (1 C), 15.57 (1 C), 36.69 (1 C), 42.92 (1 C), 50.51 (1 C), 61.77 (1 C), 73.49 (1 C), 100.44 (1 C), 111.89 (1 C), 114.39 (1 C), 122.52 (1 C), 128.54 (1 C), 128.79 (1 C), 129.33 (1 C), 139.33 (1 C), 140.20 (1 C), 145.43 (1 C), 149.86 (1 C), 154.32 (1 C), 156.11 (1 C), 159.47 (1 C), 172.27 (1 C); ESI-MS, m/z: 391.3 $[M-H]^-$. Anal. Calcd for $C_{22}H_{20}N_2O_5$: C, 67.34; H, 5.14; N, 7.14. Found: C, 67.17; H, 5.15; N, 7.15.

4.4. General procedure for the preparation of phosphoric acid diester and triester 7-methylhomocamptothecin-10-yl ester (24a–h)

To a solution of 7-methy-10-hydroxyhomocamptothecin (39.2 mg, 0.1 mmol) in pyridine (16 mL), $POCl_3$ (0.1 mL, 1.1 mmol) was added dropwise while held in an ice bath with stirring. The stirring was continued at ambient temperature for 1.5 h and alcohols or phenol (10.0 mmol) were added. The mixture was stirred for an additional 1.5 h and water (0.1 mL) was added, the resulted mixture was stirred at ambient temperature for 1 h and then evaporated to dryness. The residue was purified by chromatography over silica gel (5% MeOH/CH_2Cl_2) to afford diester and triester.

4.4.1. Phosphoric acid ethyl ester 7-methylhomocamptothecin-10-yl ester (24a)

Yellow solid, mp >300 °C. ^{1}H NMR (500 MHz, DMSO-d_6, δ_{ppm}): 0.87 (t, 3H, J = 7.1 Hz), 1.11–1.40 (m, 2H), 1.85 (q, 2H, J = 7.5 Hz), 2.67 (s, 3H), 3.14–3.52 (q, 2H ,J = 14.3 Hz), 3.85 (q, 2H, J = 7.1 Hz), 5.20 (s,2H), 5.41–5.52 (q, 2H, J = 15.1 Hz), 6.08 (s, 1H), 7.32 (s, 1H), 7.72 (d, 1H, J = 6.5 Hz), 7.87 (s, 1H), 8.03 (d, 1H, J = 9.0 Hz); MS-ESI m/z: 499.6 $[M^-]^-$.

4.4.2. Phosphoric acid benzyl ester 7-methylhomocamptothecin-10-yl ester (24b)

Yellow solid, mp >300 °C. ^{1}H NMR (500 MHz, DMSO-d_6, δ_{ppm}): 0.87 (t, 3H, J = 7.0 Hz), 1.30 (s, 2H), 1.84 (q, 2H, J = 7.4 Hz), 2.61 (s, 3H), 3.06–3.49 (q, 2H,J = 14.2 Hz), 5.20 (s, 2H), 5.40–5.51 (q, 2H, J = 15.0 Hz), 6.06 (s, 1H), 7.12–7.46 (m, 7H), 7.73 (s, 1H), 7.84 (s, 1H), 8.02 (s, 1H); MS-ESI m/z: 561.5 $[M^-]^-$.

4.4.3. Phosphoric acid diethyl ester 7-methylhomocamptothecin-10-yl ester (24c)

Yellow solid, mp >300 °C. ^{1}H NMR (500 MHz, DMSO-d_6, δ_{ppm}): 0.87 (t, 3H, J = 7.5 Hz), 1.29–1.32 (m, 6H), 1.87 (q, 2H, J = 7.3 Hz), 2.75 (s, 3H), 3.05–3.49 (q, 2H, J = 13.9 Hz), 4.21–4.27 (m, 4H), 5.30 (s, 2H), 5.39–5.55 (q, 2H, J = 15.1 Hz), 6.02 (s, 1H), 7.39 (s, 1H), 7.75 (d, 1H, J = 6.6 Hz), 7.80 (s, 1H), 8.21 (d, 1H, J = 9.2 Hz); MS-ESI m/z: 528.3 $[M^-]^-$; ^{31}P NMR (500 MHz, DMSO-d_6, δ_{ppm}): −5.27.

4.4.4. Phosphoric acid diisopropyl ester 7-methylhomocamptothecin-10-yl ester (24d)

Yellow solid, mp >300 °C. ^{1}H NMR (500 MHz, DMSO-d_6, δ_{ppm}): 0.87 (t, 3H, J = 7.4 Hz), 1.21–1.34 (m, 12H), 1.86 (q, 2H, J = 7.3 Hz), 2.74 (s, 3H), 3.04–3.48 (q, 2H, J = 13.5 Hz), 4.73–4.77 (m, 4H), 5.30 (s, 2H), 5.37–5.53 (q, 2H, J = 15.0 Hz), 6.03 (s, 1H), 7.38 (s, 1H), 7.75 (d, 1H, J = 6.3 Hz), 7.97 (s, 1H), 8.20 (d, 1H, J = 9.0 Hz); MS-ESI m/z: 555.5 $[M^-]^-$.

4.4.5. Phosphoric acid dibutyl ester 7-methylhomocamptothecin-10-yl ester (24e)

Yellow solid, mp >300 °C. ^{1}H NMR (500 MHz, DMSO-d_6, δ_{ppm}): 0.86–0.89 (m, 6H), 0.87 (t, 3H, J = 7.3 Hz), 1.34–1.38 (m, 4H), 1.87 (q, 2H, J = 7.4 Hz), 2.75 (s, 3H), 3.04–3.49 (q, 2H, J = 14.1 Hz), 4.16–4.20 (m, 4H), 5.30 (s, 2H), 5.39–5.55 (q, 2H, J = 15.0 Hz), 6.03 (s, 1H), 7.39 (s, 1H), 7.75 (d, 1H, J = 6.5 Hz), 7.98 (s, 1H), 8.20 (d, 1H, J = 9.3 Hz); ^{13}C NMR (DMSO-d_6): 8.47 (1C), 13.80 (2C), 15.66 (1C), 18.62 (2C), 32.13 (2C), 36.77 (1C), 42.84 (1C), 50.51 (1C), 61.77 (1C), 68.43 (2C), 73.24 (1C), 100.00 (1C), 120.19 (1C), 121.65 (1C), 123.04 (1C), 127.37 (1C), 129.45 (1C), 129.96 (1C), 140.81 (1C), 141.48 (1C), 145.24 (1C), 147.13 (1C), 152.05 (1C), 156.14 (1C), 159.41 (1C), 172.20 (1C); ^{31}P NMR (500 MHz, DMSO-d_6, δ_{ppm}): −5.38; MS-ESI m/z: 583.4 $[M^-]^-$. Anal. Calcd for $C_{30}H_{37}N_2O_8$ P: C, 61.64; H, 6.38; N, 4.79. Found: C, 61.49; H, 6.39; N, 4.80.

4.4.6. Phosphoric acid dipentyl ester 7-methylhomocamptothecin-10-yl ester (24f)

Yellow solid, mp >300 °C. ^{1}H NMR (500 MHz, DMSO-d_6, δ_{ppm}): 0.81–0.85 (m, 6H), 0.87 (t, 3H, J = 7.4 Hz), 1.25–1.32 (m, 8H), 1.62–1.66 (m, 4H), 1.86 (q, 2H, J = 7.5 Hz), 2.74 (s, 3H), 3.04–3.49 (q, 2H, J = 13.8 Hz), 4.16–4.20 (m, 4H), 5.30 (s, 2H), 5.39–5.55 (q, 2H, J = 15.1 Hz), 6.03 (s, 1H), 7.39 (s, 1H), 7.75 (d, 1H, J = 6.6 Hz), 7.98 (s, 1H), 8.20 (d, 1H, J = 9.2 Hz); ^{13}C NMR (DMSO-d_6): 8.58 (1 C), 14.17 (2C), 15.33 (1C), 22.00 (2C), 27.49 (2C), 29.78 (2C), 36.65 (1C), 42.77 (1C), 50.48 (1C), 61.71 (1C), 68.83 (2C), 73.50 (1C), 99.92 (1C), 113.41 (1C), 122.84 (1C), 124.18 (1C), 128.63 (1C), 129.84 (1C), 132.10 (1C), 140.02 (1C), 145.20 (1C), 146.15 (1C), 149.24 (1C), 151.99 (1C), 156.21 (1C), 159.42 (1C), 172.20 (1C); ^{31}P NMR (500 MHz, DMSO-d_6, δ_{ppm}): −5.58; MS-ESI m/z: 611.8 $[M^-]^-$. Anal. Calcd for $C_{32}H_{41}N_2O_8$ P: C, 62.73; H, 6.75; N, 4.57. Found: C, 61.55; H, 6.76; N, 4.60.

4.4.7. Phosphoric acid dicetyl ester 7-methylhomocamptothecin-10-yl ester (24g)

Yellow solid, mp >300 °C. ^{1}H NMR (500 MHz, DMSO-d_6, δ_{ppm}): 0.83–0.88 (m, 6H), 0.87 (t, 3H, J = 7.3 Hz), 1.15–1.27 (m, 52H), 1.62–1.63 (m, 4H), 1.85 (q, 2H,J = 7.2 Hz), 2.75 (s, 3H), 3.05–3.49 (q, 2H, J = 13.9 Hz), 4.16–4.18 (m, 4H), 5.29 (s, 2H), 5.40–5.55 (q, 2H, J = 15.0 Hz), 6.03 (s, 1H), 7.39 (s, 1H), 7.74 (d, 1H, J = 6.2 Hz), 7.97 (s, 1H), 8.20 (d, 1H, J = 9.1 Hz); MS-ESI m/z: 611.8 $[M^-]^-$.

4.4.8. Phosphoric acid di(2,2,2-trifluoro-ethyl) ester 7-methylhomocamptothecin-10-yl ester (24h)

Yellow solid, mp >300 °C. ^{1}H NMR (500 MHz, DMSO-d_6, δ_{ppm}): 0.86 (t, 3H, J = 7.3 Hz), 1.86 (q, 2H, J = 7.4 Hz), 2.75 (s, 3H), 3.05–3.50 (q, 2H, J = 13.9 Hz), 4.97–5.04 (m, 4H), 5.29 (s, 2H), 5.40–5.55 (q, 2H, J = 15.0 Hz), 6.03 (s, 1H), 7.39 (s, 1H), 7.74 (d, 1H, J = 6.5 Hz), 7.97 (s, 1H), 8.20 (d, 1H, J = 9.0 Hz); MS-ESI m/z: 920.8 $[M^-]^-$; ^{31}P NMR (500 MHz, DMSO-d_6, δ_{ppm}): −6.80.

4.5. General procedure for the preparation of phosphoric acid triester 7-methylhomocamptothecin-10-yl ester with phosphorous trialkyl esters (24c–f, 24h)

Iodine (63.5 mg, 0.3 mmol) was added at 0 °C to a solution of trialkylphosphite in dry CH_2Cl_2 (50 mL). After 10 min at 0 °C, the solution was allowed to warm up to room temperature and added dropwise to a solution of 7-methy-10-hydroxyhomocamptothecin (39.2 mg, 0.1 mmol) and DMAP (36.6 mg, 0.3 mmol) in dry CH_2Cl_2 (100 mL) at 0 °C. Then this solution was warmed up to room temperature and was washed with water and brine. The organic layer was dried over sodium sulfate and concentrated under reduced pressure giving crude materials which were purified by chromatography over silica gel (5% MeOH/CH_2Cl_2) to afford trimester with the yield from 84.4% to 96.0%.

4.6. DNA topoisomerase I activity assays

Camptothecin was obtained from the company of Tianzunzezhong in China. Topo I (calf thymus), buffer, BSA, loading buffer and supercoiled DNA pBR322 were all from TaKaRa Biotechnology Co., Ltd.

All reactions were carried out in 20 μL volumes (16 μL double distilled water, 2 μL DNA TopoI buffer, 2 μL 0.1% BSA) including 0.25 μg supercoiled DNA, 0.5 U Topo I with or without drug. The reaction was incubated at 37 °C for 15 min. Reactions were stopped by adding SDS (0.5% final concentration). To the reaction mixtures, 3.5 μL 6× loading buffer (0.1 mM EDTA, 7% Glycerol, 0.01% Xylene Cyanol FF, Bromopenol Blue 0.01%) was added. The mixtures were electrophoresed in 0.8% agarose gel in TAE buffer for 40 min at 120 V. The gel was stained with ethidium bromide at room temperature and photographed with UV transilluminator.

4.7. Cytotoxicity

Cytotoxicity assays are performed on human lung cancer (A549), breast cancer (MDA-MB-435) and colon cancer (LOVO) cell lines. Cells (6000–10,000) in 100 μL culture medium per well were seeded into 96-well microtest plates (Falcon, CA). Cells were treated in triplicate with gradient concentration of test drugs and incubated at 37 °C for 72 h. For three cell lines, the microculture tetrazolium [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT; Sigma, St. Louis, MO] assay was performed to measure the cytotoxic effects. The drug concentration required for 50% growth inhibition (IC_{50}) of tumor cells was determined from the dose–response curve.

4.8. In vivo antitumor activities

The in vivo antitumor activity of compounds **24e** and **24f** was evaluated and IRT was used as reference drug. BALB/C nude male mice (Certificate SCXK2003-0003, weighing 18–20 g) were obtained from Shanghai Experimental Animal Center, Chinese Academy of Sciences. A549 lung cancer cell suspensions were implanted subcutaneously into the right axilla region of mice. Treatment was begun when implanted tumors had reached a volume of about 100–300 mm^3 (after 17 days). The animals were randomized into appropriate groups (six animals/treatment and eight animals for the control group) and administered by ip injection once on day 17 after implantation of cells. Tumor volumes were monitored by caliper measurement of the length and width and calculated using the formula of $TV = 1/2 \times a \times b^2$, where a is the tumor length and b is the width. Tumor volumes and body weights were monitored every 4 days over the course of treatment. Mice were sacrificed on day 35 after implantation of cells and tumors were removed and recorded for analysis.

4.9. Solution stability

A modified HPLC method was adapted as follows for the determination of compounds **24e** and **24f**.[19] To 70 μL fractions of water solution in polystyrene tubes and preincubated at 37 °C for 5 min, was

added a 430 μL solution of the drug in dimethyl sulfoxide (DMSO, 2 mM), and the samples were incubated at 37 °C. At defined times the solution was analyzed and samples were run on a 5 μm Nucleosil C18 column (4.6 × 250 mm) at 25 °C with a flow rate of 1 mL/min of an isocratic eluent composed of methanol/water (60:40, v/v), and the eluted analytes were detected at 254 nm.

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