Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry 12 (2004) 3339–3344
Bioorganic & Medicinal Chemistry

Antitumor agents. Part 227: Studies on novel 4′-*O*-demethyl-epipodophyllotoxins as antitumor agents targeting topoisomerase II ☆

Zhiyan Xiao,[a] Kenneth F. Bastow,[a] John R. Vance[b] and Kuo-Hsiung Lee[a,*]

[a]*Natural Products Laboratory, School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7360, USA*
[b]*Plantaceutica Inc., Research Triangle Park, NC 27709-2060, USA*

Received 27 August 2003; accepted 23 March 2004
Available online 6 May 2004

Abstract—Eight novel epipodophyllotoxin derivatives (**6**–**13**), which were designed to overcome drug resistance and enhance topoisomerase II inhibition, were synthesized and evaluated. Two of these compounds (**7** and **8**) showed better preclinical activity profiles, including cell growth inhibition, cell killing, and in vitro topoisomerase II inhibition, as compared to the prototype molecule etoposide (**1**). They also retained the superior drug-resistance profile of GL-331 (**4**), an epipodophyllotoxin derivative currently in clinical evaluation.

1. Introduction

Etoposide (**1**) and teniposide (**2**) are semisynthetic derivatives of podophyllotoxin (**3**), a bioactive component of *Podophyllum peltatum* L (Fig. 1). Although podophyllotoxin is known as an antimicrotubule agent, etoposide and teniposide target DNA topoisomerase II.[2,3] Both etoposide and teniposide are clinically useful against various cancers, including small-cell lung cancer, testicular carcinoma, lymphoma, and Kaposi's sarcoma;[4,5] however, their therapeutic uses are often hindered by problems such as acquired drug-resistance. To obtain better therapeutic agents, extensive synthetic efforts have been devoted to overcome drug-resistance and improve topoisomerase II inhibition.

Molecular area-oriented chemical modification of podophyllotoxin has revealed structural features critical for the topoisomerase II inhibition: (1) the 4β-configuration is essential with various substitution accommodated at C_4; (2) the free 4′-hydroxy is crucial; (3) the *trans*-lactone D ring with 2α, 3β configuration is very important; (4) the dioxolane A ring is optimal; and (5) the free rotation

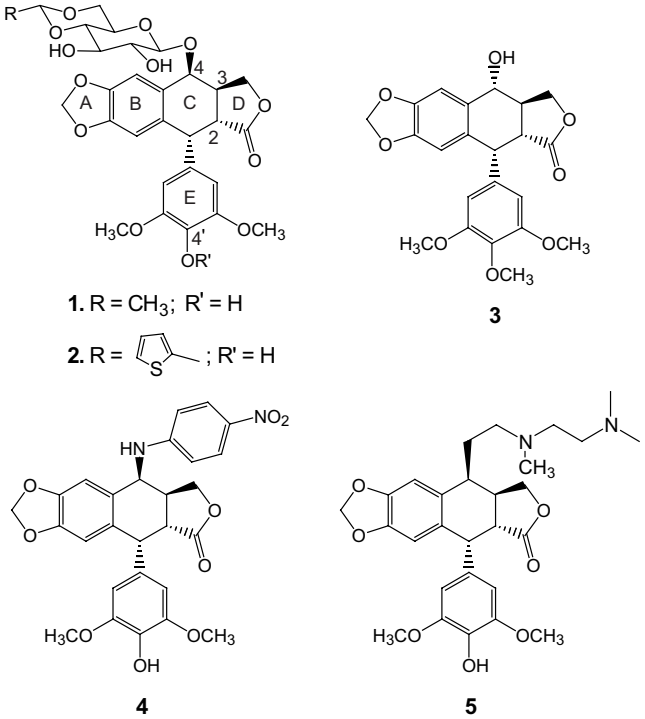


Figure 1. Structures of etoposide (**1**), teniposide (**2**), podophyllotoxin (**3**), GL-331 (**4**), and TOP-53 (**5**).

☆ For Part 226, see Ref. 1.

* Corresponding author. Tel.: +1-919-962-0066; fax: +1-919-966-3893; e-mail: khlee@unc.edu

0968-0896/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2004.03.067

of ring E is required.[6] Such structure–activity relationships (SAR) unambiguously demonstrate that C_4 is the only molecular area tolerable to significant structural diversification.

Recently, two C_4 variable **1**-analogs, GL-331 (**4**)[7] and TOP-53 (**5**),[8] have been developed. GL-331 (**4**), a 4β-arylamino derivative, was more active than **1** both in vitro and in vivo,[9] and retained cytotoxicity against **1**-resistant cells.[10] It is currently under phase II clinical evaluation against several forms of cancer, especially **1**-resistant malignancies.[11] TOP-53 (**5**), a 4β-alkylated **1**-analog, was a more potent topoisomerase II inhibitor than **1**. It exhibited high activity to nonsmall cell lung cancer in both tumor cells and animal tumor models,[12] and showed nearly wild-type potency against a mutant yeast type II enzyme highly resistant to **1**.[13] Compound **5** is currently in phase I clinical trials.[6] Both GL-331 and TOP-53 showed topoisomerase II inhibitory activity, antitumor spectra, and drug-resistance profiles significantly different from those of **1**, which suggested an important role of various C_4 substitution to the activity profiles of **1**-related analogs and the feasibility of optimizing this compound class through rational C_4 modification.

This postulation coincides with the composite pharmacophore model proposed by MacDonald et al.[14] and the comparative molecular field analysis (CoMFA) models generated by us.[15,16] Both models indicated that the C_4 molecular area could accommodate considerable structural diversity. The CoMFA model further demonstrated that bulky substituents at C_4 might be favorable for topoisomerase II inhibition. Accordingly, we designed several novel 4′-*O*-demethyl-epipodophyllotoxin derivatives (**6**–**13**, Scheme 1) bearing bulky tails at the C_4 side chain with the aim to overcome drug resistance and enhance topoisomerase II inhibition simultaneously. Because it was previously observed that the linking groups to the C_4 atom might be important for the activity of this compound class,[17] different types of C_4 linkages, including *p*-carbonyl anilino, *p*-amino anilino, amino, and carbonyl, were explored. For most compounds, protected α-amino acids were introduced to modulate the water-solubility of this compound class and potentially address another problem associated with **1** and **2**, poor water-solubility.

2. Results

Compounds **6**–**13** were first evaluated for their inhibitory activity against multiple tumor cell lines (Table 1). Compounds **7**, **8**, and **9** showed cell growth inhibitory activity (expressed as ED_{50}) superior or comparable to that of **1** and GL-331 against the tested cell lines. Notably, they retained inhibitory activity against the KB/7d **1**-resistant tumor cell line, and thus shared the superior drug-resistance profile of GL-331.

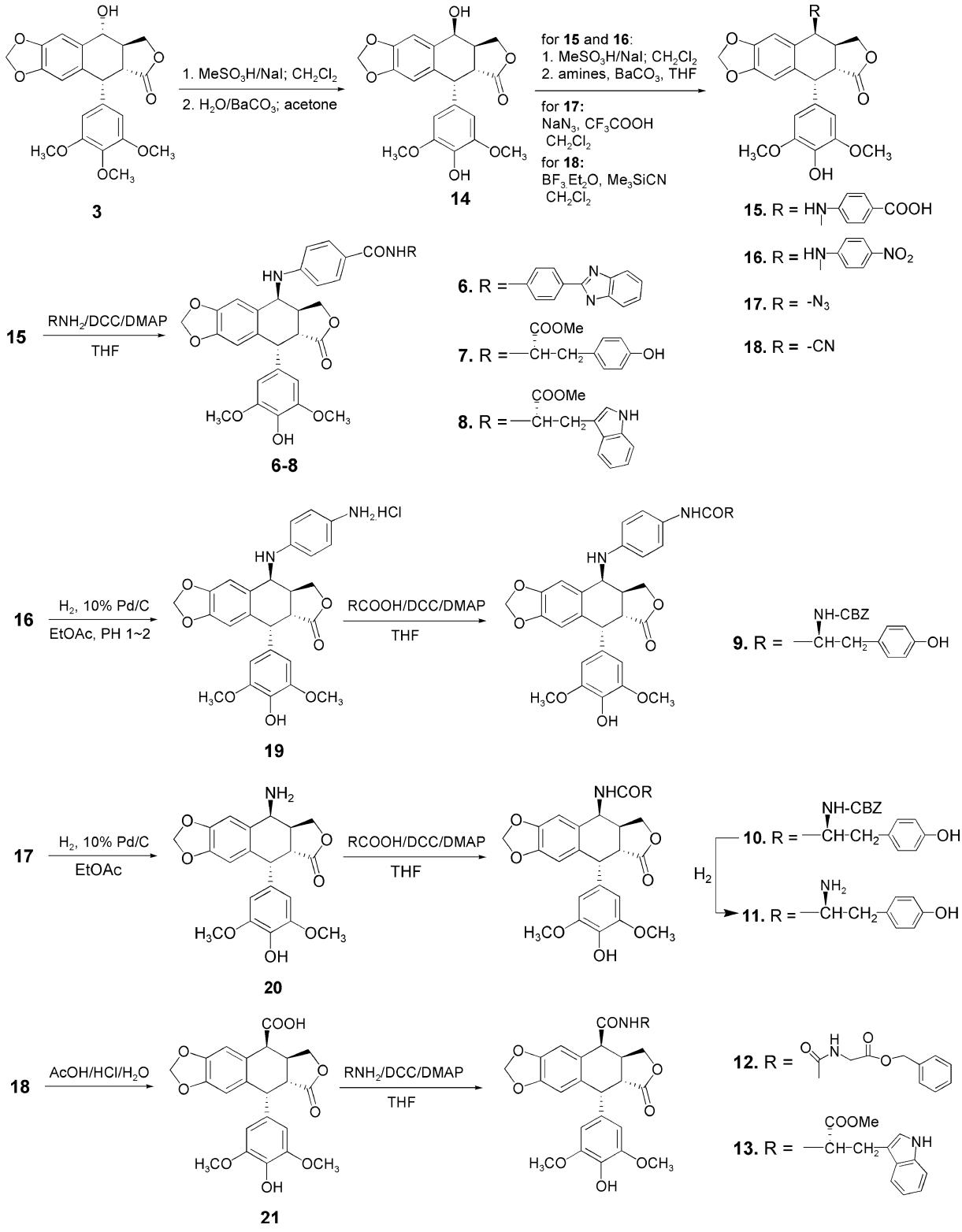
In the cytotoxicity assay (Table 1, data given as LD_{50}), compound **7** was more potent than GL-331 against MCF-7, KB, and KB-7d cells, but compound **9** was inactive. Compounds **7** and **8** were also examined with a clonogenic protocol at a concentration of 5 μM (Table 2). Both compounds (especially **7**) were much more cytotoxic than **1** and GL-331 against KB cells. Impressively, a 30 min exposure to compound **7** at 5 μM was sufficient to kill almost all treated cells. However, the cell killing effect seemed to be dose-dependent. No significant drug effects were observed for either **7** or **8** after a 30 min treatment at 0.5 μM (data not shown).

Because topoisomerase II is the pharmacological target of clinical relevance, mechanistic approaches targeting the enzyme should be more appropriate for the evaluation of these analogs. Both in vitro topoisomerase II inhibition and cellular protein-linked DNA breakage (PLDB) induction assays were applied to further assess compounds **6**–**13**. When all compounds were tested at the 50 μg/mL concentration, compounds **7**, **10**, and **11** showed comparable in vitro inhibition of topoisomerase II catalytic activity to **1** and GL-331 (Table 1). The minimum concentrations for **7** and **8** to induce detectable linear DNA were determined as 5 μM, the same concentration at which **7** and **8** showed significant cell killing activity (Table 2), while those for **1** and GL-331 were 20 and 5 μM, respectively. In the cellular protein–DNA complex formation assay, only compounds **7**, **8**, and **10** induced marked levels of cellular protein-linked DNA breaks at the tested condition, although none of them were superior to etoposide or GL-331 (Table 1).

The most promising compound, **7**, was further examined in parallel with GL-331 for its ability to induce double-strand (ds) DNA breaks and reduce KB cell colonies after short exposure (Fig. 2). Although the induction of ds DNA breaks did not correlate with cytotoxicity for either GL-331 or **7** at a concentration of 5 μM, compound **7** was superior to GL-331 in both assays.

3. Discussion and conclusion

The results in Table 1 revealed preliminary structure–activity relationships (SAR) for the tumor cell growth inhibitory activity of these novel epipodophyllotoxins. The 4β-anilino moiety at the C_4 position might be important for inhibition against tumor cell growth (GL-331 and **7**–**9** vs **10**–**13**). Extending the 4β-anilino ring with bulky groups containing another aryl ring might retain the superior activity, particularly the drug-resistance profile, of the 4β-arylamino analogs (GL-331 vs **7**–**9**). However, the spacer (e.g., distance and linkage) between the aryl rings might be critical (**6** vs **7**–**9**). Deprotection to free the amine residue markedly reduced activity (**10** vs **11**). The structural requirement for topoisomerase II inhibition was less obvious according to the available data. Although these SAR postulates are rudimentary, the good activity profiles of compounds **7** and **8** support the molecular design of extending the 4β-anilino moiety with bulky tails.



Scheme 1. Preparation of compounds **6**–**13**.

Consistent with the previous observations,[18] in vitro topoisomerase II inhibition correlated reasonably well with the ability to induce cellular protein-linked DNA breaks (PLDB). (The only exception, compound **11**, failed to induce significant amount of PLDB and also showed no marked inhibition of cell growth. Because it was much more polar than the other analogs, poor cellular uptake might be responsible for the lack of activity.) The in vitro topoisomerase II inhibitory activity of these compounds did not correlate with their inhibitory activity against cell growth. In spite of their weak inhibition against cell growth, compounds **10** and

Table 1. Biological evaluation of compounds **6–13**

Compound	In vitro topo II inhibition[a]	% PLDB formation[b]	ED_{50} (μM)[c]				LD_{50} (μM)[c]			
			A549[d]	MCF-7	KB	KB-7d	A549	MCF-7	KB	KB-7d
1	P	100	**5.2**	**>20**	**0.3**	**17.2**	>50	21	49.0	>50
4	P	244	**0.7**	**12.0**	**0.3**	**1.9**	6.6	5.3	9.9	<50
6	ND	ND	ND	ND	>25	ND	ND	ND	ND	ND
7	P	76	**2.4**	**4.5**	**0.7**	**4.5**	12.5	<3.1	1.7	<20
8	WP	58	ND	ND	**0.7**	**3.5**	ND	ND	ND	ND
9	WP	16	**0.8**	**1.9**	**0.49**	**0.41**	NA	45	>10	>50
10	P	94	15.9	17.4	3.9	11.9	ND	ND	ND	ND
11	P	8	54.3	41.7	18.8	30.2	ND	ND	ND	ND
12	ND	ND	>10	ND	11.0	16.2	ND	ND	ND	ND
13	NA	4	NA	ND	10.4	5.6	ND	ND	ND	ND

ND = not determined; NA = not active at 10 μM under test condition.

[a] All compounds were tested at 50 μg/mL. Compounds that resulted in a level of linear DNA comparable to **1** and **4** were classified as poisons (P), and those compounds that resulted in a level of linear DNA inferior to **1** and **4** were classified as weak poisons (WP).

[b] % PLDB formation, percentage of cellular protein-linked DNA breaks formed relative to **1**. All compounds were tested at 10 μg/mL.

[c] ED_{50} is the concentration that inhibits 50% cell replication after 3 days of continuous treatment; LD_{50} is the concentration that kills 50% of the cells after 30 min of treatment.

[d] Cell lines: A549 (lung adenocarcinoma); MCF-7 (breast adenocarcinoma); KB (nasopharyngeal carcinoma), and KB-7d (**1**-resistant KB sub-clone).

Table 2. Clonogenic assay of compounds **1**, **4**, **7**, and **8**

Compound[a]	Colony number	% Inhibition[b]
Control	110, 98	—
1	85, 93	0[c]
4	64, 48	46%
7	0, 1	99.5%
8	17, 21	82%

[a] All compounds were tested at 5 μM.

[b] 250 cells were plated and the plating efficiency of cells was 41%. The inhibition percentage was calculated by comparing the number of colonies surviving drug treatment with that in the control.

[c] Not markedly different from control.

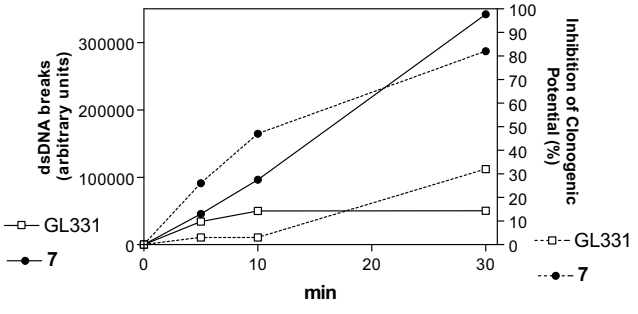


Figure 2. ds DNA break induction and KB cytotoxicity of GL-331 (**4**) and **7**. Both compounds were tested 5 μM. Double-stranded DNA breaks: KB cells were mock treated (medium change only) or treated with GL-331 (**4**) or **7**. The increases in intensities of drug-induced double-stranded DNA breaks were quantified by measuring the fluorescence intensity of the corresponding bands as compared to that in the control. Inhibition of clonogenic potential: the data were based on the colony-forming ability of cells after short treatment (30 min) with the tested compounds. The inhibition percentage was calculated by comparing the number of colonies surviving drug treatment with that in the control.

11 were characterized as topoisomerase II poisons in vitro. In contrast, the potent cell growth inhibitors, compounds **8** and **9**, were found to be only weak enzyme poisons. Other possible mechanisms of action (e.g., inhibition of tubulin polymerization) and differences in cellular uptake and chromosomal DNA breakage patterns could contribute to the lack of correlation between these parameters.

In summary, based on previous structure–activity relationship and molecular modeling studies, eight novel C_4 modified epipodophyllotoxin derivatives were designed and synthesized to overcome drug resistance and enhance topoisomerase II inhibition simultaneously. Two compounds (**7** and **8**) showed superior preclinical activity profiles to the etoposide (**1**) prototype. They were potent inhibitors of tumor cell growth and potent cytotoxic agents (Tables 1 and 2). These activities are likely to be mediated by DNA topoisomerase II according to the plasmid cleavage and PLDB induction assays (Table 1 and Fig. 2). Compounds **7** and **8** also exhibited a good drug-resistance profile similar to that of GL-331. These results agree with the hypothesis that C_4 derivation is an effective approach to optimize the activity profiles of this compound class. The preparation and biological testing of additional 4′-*O*-demethyl-epipodophyllotoxin derivatives with bulky substitutions at C_4 position are underway and will be reported upon completion of the studies.

4. Experimental

4.1. Chemistry

Compounds **6–13** were prepared from podophyllotoxin (**3**, Scheme 1) according to previously published methods. Briefly, 4′-demethyl-epipodophyllotoxin (DMEP, **14**) was synthesized from **3** stereoselectively through successive 4′-demethylation, 4-iodination with methanesulfonic acid/sodium iodide, and nucleophilic substitution with water.[19] This important intermediate was subjected to nucleophilic displacement by 4-amino

benzoic acid, *p*-nitroaniline, sodium azide, and trimethylsilyl cyanide to provide intermediates **15**, **16**, **17**, and **18**, respectively. Compound **15** was condensed with the appropriate amines in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridine (DMAP) to provide compounds **6**–**8**. Intermediates **16** and **17** underwent Pd–C catalyzed hydrogenation to afford **19** and **20**, respectively, which were subsequently condensed with the corresponding acids to give compounds **9** and **10**. Deprotection of compound **10** through catalytic hydrogenation provided compound **11**. Intermediate **18** was easily hydrolyzed to the carboxylic acid **21** in the presence of acetic acid and hydrochloric acid.[20] Condensation of **21** with the appropriate amines provided compounds **12** and **13**, respectively. The structures of compounds **6**–**13** were confirmed by spectroscopic and analytical data.

Compound **6**: yield 45%; mp 152–154 °C (dec); $[\alpha]_D^{25}$ −36.0 (*c* 0.05, acetone); IR (film) 1727 (lactone and amide) 1475, 1455, 1273 (aromatic C=C) cm^{-1}; MS *m*/*e*: 709 $[M-1]^+$; ^1H NMR ($CDCl_3$) δ 7.52 (d, $J = 8.7$ Hz, 2H, 2″,6″-H), 7.43 (d, $J = 8.7$ Hz, 2H, 2′,6′-H), 7.41 (m, 2H, 4‴,7‴-H), 6.72 (s, 1H, 5-H), 6.63 (d, $J = 8.7$ Hz, 2H, 3′,5′-H), 6.62 (m, 5‴,6‴-H), 6.54 (s, 1H, 8-H), 6.53 (d, $J = 8.7$ Hz, 2H, 3″,5″-H), 6.33 (s, 2H, 2′,6′-H), 5.90 (dd, 2H, –OCH_2O–), 4.74 (m, 1H, 4-H), 4.60 (d, $J = 4.8$ Hz, 1H, 1-H), 4.37 (t, $J = 7.8$ Hz, 1H, 11-H), 3.78 (m, 1H, 11-H), 3.80 (s, 6H, 3′,5′-OCH_3), 3.50 (m, 1H, 2-H), 3.06 (m, 1H, 3-H).

Compound **7**: yield 31%; mp 203 °C (dec); $[\alpha]_D^{25}$ −183.3 (*c* 0.03, acetone); IR (film) 1737 (lactone) 1727 (amide and ester) 1462, 1445, 1427 (aromatic C=C) 1217 (phenol) cm^{-1}; MS *m*/*e*: 696 $[M]^+$; ^1H NMR (acetone) δ 8.38 (s, 1H, –OH), 7.71 (d, $J = 8.7$ Hz, 2H, 3″,5″-H), 7.51 (d, $J = 8.1$ Hz, 1H, –NH), 7.21 (s, 1H, –OH), 7.12 (d, $J = 8.7$ Hz, 2H, 2′,6′-H), 6.82 (s, 1H, 5-H), 6.75 (d, $J = 8.7$ Hz, 2H, 3′,5′-H), 6.74 (d, $J = 8.7$ Hz, 2H, 2″,6″-H), 6.52 (s, 1H, 8-H), 6.40 (s, 2H, 2′,6′-H), 5.96 (dd, 2H, –OCH_2O–), 5.03 (m, 1H, 1-H), 4.80 (m, 1H, –CO–CH–N–), 4.55 (d, $J = 4.5$ Hz, 1H, 4-H), 4.39 (t, $J = 8.1$ Hz, 1H, 11-H), 3.85 (t, $J = 8.1$ Hz, 1H, 11-H), 3.70 (s, 6H, 3′,5′-OCH_3), 3.25–3.02 (m, 4H, 2, 3-H, –CO–CH–CH_2–).

Compound **8**: yield 91%; mp 177–179 °C (dec); $[\alpha]_D^{25}$ −48.0 (*c* 0.05, acetone); IR (film) 1736 (lactone) 1727 (amide and ester) 1478, 1461, 1433 (aromatic C=C) 1217 (phenol) cm^{-1}; MS *m*/*e*: 720 $[M+1]^+$; ^1H NMR (acetone, D_2O exchange) δ 7.67 (d, $J = 8.7$ Hz, 2H, 3″, 5″-H), 7.58 (d, $J = 8.1$ Hz, 1H, 1′-H), 7.35 (d, $J = 8.1$ Hz, 1H, 2′-H), 7.22 (s, 1H, –CO–NH–), 7.02 (m, 4H, 4′-7′-H), 6.77 (s, 1H, 5-H), 6.73 (d, $J = 8.7$ Hz, 2H, 2″,6″-H), 6.49 (s, 1H, 8-H), 6.35 (s, 2H, 2′,6′-H), 5.94 (d, $J = 2.1$ Hz, 2H, –OCH_2O–), 5.00 (m, 1H, 1-H), 4.90 (m, 1H, –CO–CH–N–), 4.54 (d, $J = 4.8$ Hz, 1H, 4-H), 4.37 (t, $J = 7.8$ Hz, 1H, 11-H), 3.78 (t, $J = 7.8$ Hz, 1H, 11-H), 3.71 (s, 6H, 3′,5′-OCH_3), 3.64 (s, 3H, $COOCH_3$), 3.43–3.16 (m, 4H, 2, 3-H, –NH–CH–CH_2–).

Compound **9**: yield 48%; mp 192–195 °C; $[\alpha]_D^{25}$ −76.0 (*c* 0.05, acetone); IR (film) 1738 (lactone, with shoulder) 1475, 1450, 1420 (aromatic C=C) 1216 (phenol) cm^{-1}; MS *m*/*e*: 787 $[M]^+$; ^1H NMR (acetone) δ 9.01 (s, 1H, –OH), 7.40 (d, $J = 8.7$ Hz, 2H, 3″,5″-H), 7.32 (m, 5H, 2‴-6‴-H), 7.12 (d, $J = 8.4$ Hz, 2H, 2′,6′-H), 6.82 (s, 1H, 5-H), 6.75 (d, $J = 8.4$ Hz, 2H, 3′,5′-H), 6.69 (d, $J = 8.7$ Hz, 2H, 2″,6″-H), 6.52 (s, 1H, 8-H), 6.39 (s, 2H, 2′,6′-H), 5.96 (s, 2H, –OCH_2O–), 5.03 (d, $J = 6.9$ Hz, 2H, –OCH_2–Ph), 4.88 (m, 1H, 1-H), 4.55 (d, $J = 4.5$ Hz, 1H, 4-H), 4.41 (m, 1H, –CO–CH–N–), 4.39 (t, $J = 8.1$ Hz, 1H, 11-H), 3.91 (t, $J = 8.1$ Hz, 1H, 11-H), 3.70 (s, 6H, 3′,5′-OCH_3), 3.31–3.10 (m, 4H, 2, 3-H, –CO–CH–CH_2–), 2.92 (m, 1H, 3-H).

Compound **10**: yield 80%; mp 155–157 °C; $[\alpha]_D^{25}$ −24.0 (*c* 0.05, acetone); IR (film) 1745 (lactone), 1735 (amides) 1475, 1450, 1420 (aromatic C=C) cm^{-1}; MS *m*/*e*: 697 $[M+1]^+$; ^1H NMR (DMSO) δ 9.15 (s, 1H, –OH), 8.37 (d, $J = 8.1$ Hz, 1H, –NH), 8.23 (s, 1H, –OH), 7.46 (d, $J = 7.5$ Hz, 1H, –NH), 7.30 (m, 5H, 2′-6′-H), 7.05 (d, $J = 8.4$ Hz, 2H, 2″,6″-H), 6.65 (d, $J = 8.4$ Hz, 2H, 3′,5″-H), 6.57 (s, 1H, 5-H), 6.52 (s, 1H, 8-H), 6.24 (s, 2H, 2′,6′-H), 5.99 (d, $J = 15.3$ Hz, 2H, –OCH_2O–), 5.13 (m, 1H, 1-H), 4.96 (d, $J = 5.1$ Hz, 2H, –OCH_2–Ph), 4.50 (d, $J = 5.1$ Hz, 1H, 4-H), 4.19 (t, $J = 8.1$ Hz, 1H, 11-H), 4.11 (m, 1H, –CO–CH–N–), 3.85 (t, $J = 8.1$ Hz, 1H, 11-H), 3.63 (s, 6H, 3′,5′-OCH_3), 3.32 (m, 1H, 2-H), 3.05 (d, $J = 5.4$ Hz, 2H, –CO–CH–CH_2–), 2.82 (m, 1H, 3-H).

Compound **11**: yield 30%; mp 172–175 °C; $[\alpha]_D^{25}$ −66.0 (*c* 0.05, acetone); IR (film) 1770 (lactone), 1701 (amide), 1465, 1458, 1365 (aromatic C=C), 1122 (phenol) cm^{-1}; MS *m*/*e*: 563$[M+1]^+$; ^1H NMR (CD_3OD) δ 7.04 (d, $J = 8.4$ Hz, 2H, 2″,6″-H), 6.68 (d, $J = 8.4$ Hz, 2H, 3″,5″-H), 6.32 (s, 1H, 5-H), 6.20 (s, 2H, 2′,6′-H), 6.19 (s, 1H, 8-H), 5.93 (dd, $J = 2.4$, 0.9 Hz, 2H, –OCH_2O–), 4.98 (d, $J = 2.1$ Hz, 1H, 1-H), 4.42 (d, $J = 5.1$ Hz, 1H, 4-H), 4.23 (t, $J = 8.1$ Hz, 1H, 11-H), 3.96 (t, $J = 8.1$ Hz, 1H, 11-H), 3.70 (s, 1H, –CO–CH–N–), 3.60 (s, 6H, 3′,5′-OCH_3), 3.18 (m, 1H, 2-H), 2.88 (m, 1H, 3-H).

Compound **12**: yield 35%; mp 131–133 °C; $[\alpha]_D^{25}$ −100.0 (*c* 0.2, acetone); IR (film) 1740, 1735 (lactone and amide) 1455, 1365 (aromatic C=C) 1217 (phenol) cm^{-1}; MS *m*/*e*: 574 $[M-1]^+$; ^1H NMR ($CDCl_3$) δ 7.37 (m, 5H, 2″-6″ H), 7.08 (s, 1H, 5-H), 6.39 (s, 1H, 8-H), 6.07 (s, 2H, 2′,6′-H), 5.91 (d, $J = 1.8$ Hz, 2H, –OCH_2O–), 5.17 (d, $J = 2.7$ Hz, 1H, 1-H), 4.11 (m, 5H, 4, 11-H, –NH–CH_2–), 3.72 (s, 6H, 3′,5′-OCH_3), 3.13 (m, 1H, 3-H), 2.80 (m, 1H, 2-H).

Compound **13**: yield 41%; MS *m*/*e*: 627 $[M-1]^+$; ^1H NMR ($CDCl_3$) δ 7.46 (d, $J = 7.5$ Hz, 1H, 1′-H), 7.27 (d, $J = 7.8$ Hz, 1H, 2′-H), 7.18 (s, 1H, 5-H), 7.07 (m, 5H, 8-H, 4′-7′-H), 6.88 (s, 2H, 2′, 6′-H), 5.99 (d, $J = 9.9$ Hz, 2H, –OCH_2O–), 4.88 (m, 2H, 1-H, –NH–C*H*–CO–), 4.12–3.70 (m, 3H, 4, 11-H), 3.61 (s, 6H, 3′,5′-OCH_3), 3.60 (s, 3H, $COOCH_3$), 3.30–3.17 (m, 4H, 2,3-H, –NH–CH–CH_2–).

4.2. Biology

Cell growth inhibition assay. Cell growth inhibition was assayed using the sulforhodamine B (SRB) protocol

developed by Rubinstein et al.[21] Drug exposure was for 3 days, and the ED_{50} value was interpolated from dose–response data.

Clonogenic assay. The detailed protocol for clonogenic assay has been described previously.[22] Briefly, 250 freshly trypsinized KB cells were exposed to drug at various concentrations for 30 min, and then cells were immediately plated and left undisturbed for 12 days. Colonies were fixed with formalin, stained with toluidine blue, and then scored. The plating efficiency and percentage of total cells forming colonies were calculated. LD_{50} concentrations were determined by interpolating from graphed data.

In vitro DNA topoisomerase II assay. The P4 DNA unknotting assay was carried out according to the procedure described previously.[18] Assays were performed with drug concentrations of 50 μg/mL.

K^+-SDS precipitation assay for cellular protein–DNA complexes. Stimulation of intracellular protein-associated DNA breaks was measured using a standard assay method,[23] and the procedure was described in our previous publication.[18] All compounds were tested in duplicate at 10 μg/mL.

Gel lysis for detection of cellular DNA breaks. A published gel-based semi-quantitative assay was used.[22] Briefly, KB cells were treated with drugs for various times and were then harvested, cast in agarose plugs, and de-proteinized. Gel plugs were subjected to agarose–gel electrophoresis and levels of ethidium-stained broken DNA fragments were determined using a STORM phosphor-Images (Molecular Dynamics, Sunnyvale, Calif.), and the supplied Image Plant software. The relative amounts of double-stranded DNA were compared by measuring the fluorescence intensity of the corresponding 'ds DNA fragment' bands on the gel.

Acknowledgement

This investigation was supported by a NIH grant CA 17625 awarded to K. H. Lee.

References and notes

1. Xia, Y.; Yang, Z. Y.; Xia, P.; Bastow, K. F.; Nakanishi, Y.; Nampoothiri, P.; Hackl, T.; Hamel, E.; Brossi, A.; Kuo, S. C.; Lee, K.H. *Bioorg. Med. Chem. Lett.*, **2003**, *13*, 2891.
2. Osheroff, N.; Zechiedrich, E. L.; Gale, K. C. *Bio. Essays* **1991**, *13*, 269.
3. Alton, P. A.; Harris, A. L. *Br. J. Haematol.* **1993**, *85*, 241.
4. Jardine, I. *Anticancer Agents Based on Natural Products Models*; Academic: New York, 1980; p 319.
5. Issell, B. F. *Cancer Chemother. Pharmacol.* **1982**, *7*, 73.
6. Moraes, R. M.; Dayan, F. E.; Canel, C. *Studies in Natural Products Chemistry* **2002**, *26*, 149.
7. Wang, Z. Q.; Kuo, Y. H.; Schnur, D.; Bowen, J. P.; Liu, S. Y.; Han, F. S.; Chang, J. Y.; Cheng, Y. C.; Lee, K. H. *J. Med. Chem.* **1990**, *33*, 2660.
8. Terada, T.; Fujimoto, K.; Nomura, M.; Yamashita, J.; Wierzba, K.; Yamazaki, R.; Shibata, J.; Sugimoto, Y.; Yamada, Y.; Kobunai, T.; Takeda, S.; Minami, Y.; Yoshida, K.; Yamaguchi, H. *J. Med. Chem.* **1993**, *36*, 1689.
9. Lee, K. H. *Med. Res. Rev.* **1999**, *19*, 569.
10. Chang, J. Y.; Han, F. S.; Liu, S. Y.; Wang, Z. Q.; Lee, K. H.; Cheng, Y. C. *Cancer Res.* **1991**, *51*, 1755.
11. Choy, W. Genelabs Technologies, personal communication.
12. Utsugi, T.; Shibata, J.; Sugimoto, Y.; Aoyagi, K.; Wierzba, K.; Kobunai, T.; Terada, T.; Ohhara, T.; Tsuruo, T.; Yamada, Y. *Cancer Res.* **1996**, *56*, 2809.
13. Byl, J. A. W.; Cline, S. D.; Utsugi, T.; Kobunai, T.; Yamada, Y.; Osheroff, N. *Biochemistry* **2001**, *40*, 712.
14. MacDonald, T. L.; Lehnert, E. K.; Loper, J. T.; Chow, K. C.; Ross, W. E. *DNA Topoisomerase in Cancer*; Oxford University: New York, 1991; p 119.
15. Cho, S. J.; Tropsha, A.; Suffness, M.; Cheng, Y. C.; Lee, K. H. *J. Med. Chem.* **1996**, *39*, 1383.
16. Xiao, Z.; Xiao, Y. D.; Feng, J.; Golbraikh, A.; Tropsha, A.; Lee, K. H. *J. Med. Chem.* **2002**, *45*, 2294.
17. Zhou, X. M.; Wang, Z. Q.; Chang, J. Y.; Chen, H. X.; Cheng, Y. C.; Lee, K. H. *J. Med. Chem.* **1991**, *34*, 3346.
18. Lee, K. H.; Imakura, Y.; Haruna, M.; Beers, S. A.; Thurston, L. S.; Dai, H. J.; Chen, C. H. *J. Nat. Prod.* **1989**, *52*, 606.
19. Kamal, A.; Laxman, N.; Ramesh, G. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2059.
20. Chen, Z. X.; Ma, W. Y.; Zhang, C. N. *Chin. Chem. Lett.* **2000**, *6*, 505.
21. Rubinstein, L. V.; Shoemaker, R. H.; Paull, K. D.; Simon, R. M.; Tosini, S.; Skehan, P.; Scudiero, D. A.; Monks, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1113.
22. Krishnan, P.; Bastow, K. F. *Cancer Chemother. Pharmacol.* **2001**, *47*, 187.
23. Rowe, T. D.; Chen, G. L.; Hsiang, Y. H.; Liu, L. F. *Cancer Res.* **1986**, *46*, 2021.