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Antitumor Agents 210. Synthesis and Evaluation of Taxoid–Epipodophyllotoxin Conjugates as Novel Cytotoxic Agents†

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Abstract—Five compounds composed of a taxoid (paclitaxel or cephalomannine) and a 4′-*O*-demethyl epipodophyllotoxin derivative joined by an imine linkage were prepared and evaluated as cytotoxic agents and inhibitors of mammalian DNA topoisomerase II. Compounds **12** and **14**–**16** exhibited comparable or better activity than the unconjugated epipodophyllotoxin derivatives in most tumor cell lines, and **12**, **15**, and **16** also showed enhanced activity against paclitaxel-resistant cells. Compound **13**, which contains an epipodophyllotoxin moiety at both the taxoid 2′ and 7 positions, did not stimulate protein–DNA breaks, but was 2-fold more potent than **12** and **15** and comparable to GL-331 in the topo II inhibitory assay.

Introduction

The complex diterpenoid Taxol® (paclitaxel, **1**) is an exciting new anticancer drug, which is currently in clinical use against ovarian and breast cancer. It acts by an unusual mechanism: promoting microtubule assembly. Since the discovery of paclitaxel as an antimitotic antitumor agent, its chemistry and structure–activity relationships (SAR) have been extensively studied in order to develop more potent derivatives and to overcome its low water solubility and drug resistance problems.[2,3] Research has focused on modifications at C2, C4, C7, C9, C10, and the C13 side chain, and the resulting structure–activity relationships have led to more potent paclitaxel derivatives. The 2- and 7-positions have been used frequently for prodrug and analogue modification, respectively.[4] Substitution at these positions led to different tubulin binding.[5–7] Also, selective modification of the 2′-hydroxyl led to synthesis of a series of water-soluble derivatives.[4] However, drug resistance is still a major problem associated with paclitaxel, and novel derivatives are highly desirable.

Previously, we synthesized and evaluated two compounds (**3** and **4**) that are conjugates of a camptothecin (CPT) derivative and an etoposide (**5**) analogue.[8] Both compounds induced protein-linked DNA breaks (PLDB) in a concentration dependent manner in drug treated cells. The drug induced PLDB could be mediated by both DNA topoisomerases (topo) I and II. The two conjugates were more active than CPT against CPT-resistant cells and than etoposide against etoposide-resistant cells. Over-expression of the multiple drug resistant proteins GP170 or MRP had little impact on the cytotoxicity. The compounds were equally cytotoxic as CPT in CPT-sensitive or etoposide-resistant cells and as etoposide in CPT-resistant cells. In vivo, one conjugate (**3**) was more active than both etoposide and CPT against human KB and DU-145 tumor cells in nude mice. This novel topo I and II dual inhibitory property as well as the unique antitumor action of these CPT-etoposide analogue conjugates suggests that such bi-molecular models should be further explored as anticancer drugs.

In order to develop paclitaxel analogues with multiple antitumor mechanisms or improved activity against drug resistant cells, our attention focused on the design and synthesis of a series of 2′-(4″-*O*-demethyl epipodophyllotoxin) taxoids (Scheme 1). These compounds possess an interesting bi-molecular structure: a topo II inhibitory 4′-*O*-demethyl epipodophyllotoxin is conjugated with an antimitotic taxoid at the 2′ or 7 position. This report describes the structures, synthesis, and

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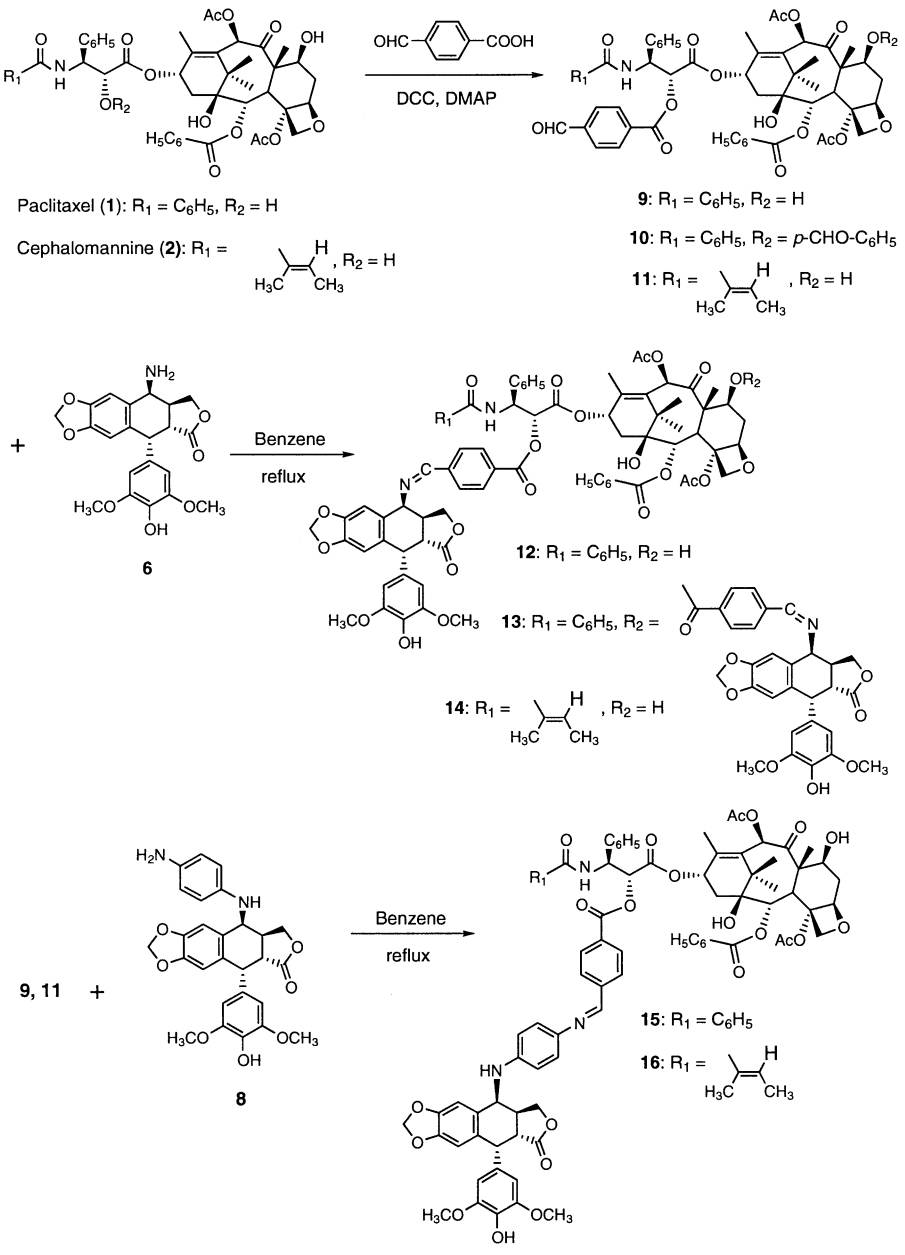

Scheme 1. Synthesis of taxoid-epipodophyllotoxin conjugates.

preliminary antitumor testing results of these compounds.

Results and Discussion

The 2′-hydroxyl of paclitaxel is more reactive than the sterically hindered 7-hydroxyl.[9–11] Therefore, a 4′-*O*-demethyl epipodophyllotoxin analogue can be introduced selectively at the 2′-position of paclitaxel via a benzaldehyde bridge. The three mono- and bis-(4-formyl)benzoyl taxoids (**9**–**11**) were synthesized by reacting paclitaxel (**1**) and cephalomannine (**2**) with 4-carboxybenzaldehyde in the presence of 1,3-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (4-DMAP) (Scheme 1), respectively. 4′-*O*-Demethyl-4β-amino-4-desoxypodophyllotoxin, **6**, was synthesized according to a literature method.[12] 4′-*O*-Demethyl-4β-(*p*-aminoanilino)-4-desoxypodophyllotoxin, **8**, was synthesized by treating podophyllotoxin with trimethylsilyl iodide (TMSI) in methylene chloride, followed by substitution with *p*-nitroaniline in the presence of barium carbonate,[13] and finally, by hydrogenation of the resulting nitro compound. Syntheses of the desired conjugates (**12**–**16**) were accomplished by reaction of **9**–**11** with **6** or **8** in refluxing benzene to form an imine linkage (Scheme 1). The structures of these conjugates were confirmed unambiguously from NMR and mass spectral data. The detailed assignments of the NMR signals are listed in Table 1.

The newly synthesized taxoid-4′-*O*-demethyl epipodophyllotoxin conjugates were evaluated in house using standard cytotoxicity assays in human tumor (HTCL) and drug-resistant (DRCL) cell lines. Reference compounds were paclitaxel (**1**), cephalomannine (**2**), and

Table 1. ^{1}H NMR assignments of taxoids and their conjugates

Proton no.	9	10	11	12	13	14	15	16
Signals belonging to taxoid portion								
2	5.68 (d, 7)	5.80 (d, 7)	5.68 (d, 7)	5.68 (d, 7)	5.69 (d, 7)	5.68 (d, 7)	5.68 (d, 7)	5.68 (d, 7)
3	4.20 (d, 8)	4.06 (d, 8)	3.81 (d, 7)	3.81 (d, 7)	4.04 (dd, 7, 2)	3.81 (d, 7)	3.82 (d, 7)	3.81 (d, 7)
5	4.98 (d, 9)	5.02 (d,9)	4.97 (d, 9)	4.97 (d, 10)	5.04 (br.d, 9)	4.96 (d, 9)	4.97 (dd, 8, 2)	4.96 (d, 9)
6α, β	1.90 (tt, 13, 3)	1.92 (m)	1.87 (m)	1.88 (br.t, 13)	1.92 (m)	1.88 (m)	1.88 (ddd, 16, 11, 2)	1.88 (br. t, 13)
7	4.45 (m)	5.81 (dd, 10, 4)	4.46 (m)	4.44 (dd, 10, 7)	5.82 (m)	4.44 (m)	4.45 (dd, 11, 7)	4.45 (m)
10	6.30 (s)	6.38 (s)	6.30 (s)	6.30 (s)	6.38 (s)	6.30 (s)	6.30 (s)	6.30 (s)
13	6.27 (t, 9)	6.25 (t, 9)	6.26 (br. t, 9)	6.26 (t, 9)	6.24 (t, 9)	6.26 (br. t, 9)	6.27 (t, 9)	6.26 (br. t, 9)
14α, β	2.14 (dd, 15, 9)	2.17 (dd, 15, 9)	2.55 (m)	2.14 (dd, 15, 9)	2.18 (m)	2.55 (m)	2.14 (dd, 15, 9)	2.55 (m)
	2.34 (dd, 15, 9)	2.38 (dd, 15, 9)		2.33 (dd, 15, 9)	2.36 (m)		2.33 (dd, 15, 9)	
16-CH_3	1.23 (s)	1.21 (s)	1.26 (s)	1.23 (s)	1.26 (s)	1.25 (s)	1.24 (s)	1.26 (s)
17-CH_3	1.14 (s)	1.18 (s)	1.15 (s)	1.14 (s)	1.19 (s)	1.14 (s)	1.14 (s)	1.15 (s)
18-CH_3	1.97 (s)	1.97 (s)	1.82 (s)	1.96 (s)	1.97 (s)	1.82 (s)	1.96 (s)	1.83 (s)
19-CH_3	1.68 (s)	1.95 (s)	1.68 (s)	1.68 (s)	1.96 (s)	1.68 (s)	1.68 (s)	1.68 (s)
20α	4.32 (d, 8.4)	4.37 (d, 8)	4.30 (d, 8)	4.31 (d, 9)	4.37 (d, 8)	4.30 (d, 8)	4.31 (d, 9)	4.29 (d, 8)
20β	4.20 (d, 8)	4.25 (d, 8)	4.20 (d, 8)	4.19 (d, 9)	4.27 (d, 8)	4.19 (d, 8)	4.20 (d, 9)	4.19 (d, 8)
2′	5.72 (d,4)	5.75 (d, 4)	5.64 (d, 3)	5.70 (d, 4)	5.70 (d, 4)	5.62 (br. s)	5.71 (d, 4)	5.65 (br. s)
3′	6.08 (dd, 10, 4)	6.09 (dd, 9, 4)	5.94 (dd, 9, 3)	6.04 (dd, 9, 4)	6.08 (dd, 9, 4)	5.92 (br. s)	6.05 (dd, 9, 4)	5.92 (br. dd, 9, 3)
3′-NH	6.97 (d, 9.6)	7.07 (d, 8)	6.55 (d, 9)	7.02 (d, 9)	[a]	6.56 (d, 9)	7.06 (d, 9)	6.61 (d, 8)
2-OBz (o)	8.13 (d, 7)	8.13 (d, 8)	8.12 (d, 8)	8.13 (d, 7)	8.14 (d, 7)	8.12 (d, 8)	8.11 (d, 7.5)	8.11 (d, 8)
2-OBz (m)	7.52 (t, 7)	7.53 (d, 8)	7.51 (t, 8)	7.52 (d, 7)	7.52 (d, 7)	7.52 (t, 8)	7.52 (d, 7.5)	7.51 (t, 8)
2-OBz (p)	7.62 (m)	7.64 (t, 8)	7.62 (t, 8)	7.62 (t, 8)	7.64 (t, 7)	7.62 (t, 8)	7.61 (t, 7.5)	7.61 (t, 8)
7-OBz (o)	—	7.93 (d, 8)	—	—	7.97 (d, 8)	—	—	—
7-OBz (m)	—	8.07 (d, 8)	—	—	7.81 (d, 8)	—	—	—
2′-OBz (o)	8.14 (d, 8)	8.15 (d, 8)	8.12 (d, 8)	8.04 (dd, 7, 2)	8.08 (d, 8)	7.85 (d, 8)	8.15 (d, 8)	8.11 (br. d, 8)
2′-OBz (m)	7.98 (d, 8)	7.97 (d, 8)	7.97 (d, 8)	7.85 (dd, 7, 2)	7.86 (d, 8)	8.01 (d, 8)	7.76 (d, 8)	7.52 (br. d, 8)
3′-Ph (o)	7.45 (d, 8)	7.48 (m)	7.40 (br.s)	7.44 (t, 8)	7.43 (d, 7)	7.40 (m)	7.43 (d, 8)	7.40 (m)
3′-Ph (m)	7.41 (t, 8)	7.42 (m)	7.40 (br.s)	7.41 (t, 8)	7.47 (t, 7)	7.40 (m)	7.41 (t, 8)	7.40 (m)
3′-Ph (p)	7.44 (t, 8)	7.35 (dd, 7, 2)	7.33 (t, 7)	7.43 (d, 8)	7.33 (br.t, 7)	7.32 (m)	7.42 (d, 8)	7.32 (m)
NBz (o)	7.76 (d, 8)	7.77 (br.d, 8)	—	7.75 (d, 8)	7.75 (d, 7)	—	7.76 (d, 8)	—
NBz (m)	7.52 (br. t, 7)	7.45 (m)	—	7.51 (t, 7)	7.47 (t, 7)	—	7.51 (t, 7)	—
NBz (p)	7.52 (br. t, 7)	7.46 (m)	—	7.52 (t, 7)	7.53 (t, 7)	—	7.52 (t, 7)	—
1-OH	1.89 (bt. t, 11, 2)	1.92 (br. s)	—	1.97 (s)	1.97 (s)	—	1.96 (s)	—
7-OH	2.54 (br. s)	—	—	2.49 (br. s)	—	—	2.50 (br. s)	—
2′-OH	—	—	—	—	—	—	—	—
4-OAc	2.46 (s)	2.49 (s)	2.43 (s)	2.44 (s)	2.49 (s)	2.41 (s)	2.43 (s)	2.41 (s)
10-OAc	2.23 (s)	2.06 (s)	2.23 (s)	2.23 (s)	2.07 (s)	2.23 (s)	2.22 (s)	2.23 (s)
2″-CH_3	—	—	1.97 (s)	—	—	1.96 (br. s)	—	1.96 (s)
3″	—	—	6.43 (br.q, 7)	—	—	6.43 (br.q, 7)	—	6.45 (br.q, 7)
4″-CH_3	—	—	1.72 (d, 7)	—	—	1.72 (d, 7)	—	1.73 (d, 7)
CHO	10.13 (s)	10.10 (s), 10.12 (s)	10.11 (s)	—	—	—	—	—
Signals belonging to epipodophyllotoxin portion								
1‴				4.61 (br. d, 4)	4.71 (2H, d, 5)	4.67 (br. s)	4.62 (d, 5)	4.61 (br. d, 4)
2‴				3.13 (m)	3.12 (2H, m)	3.13 (m)	3.14 (dd, 15, 5)	3.14 (dd, 14, 4)
3‴				3.11 (m)	3.05 (2H, m)	3.12 (m)	3.02 (tdd, 15, 7, 4)	3.04 (m)
4‴				4.71 (d, 5)	4.68 (2H, t, 4)	4.71 (d, 5)	4.73 (dd, 5, 5)	4.73 (br. s)
5‴				6.58 (s)	6.39 (2H, s)	6.59 (s)	6.80 (s)	6.80 (s)
8‴				6.39 (s)	6.60 (2H, s)	6.39 (s)	6.55 (s)	6.54 (s)
11‴				4.00, 4.27 (t, 9)	4.26 (2H, m)	4.20, 4.27 (t, 9)	4.22 (td, 11, 5)	4.01, 4.40 (t, 9)
					4.00 (2H, m)		4.00 (td, 11, 5)	
OCH_2O				5.92, 5.98 (br. s)	5.98, 5.92 (both 2H, each br.s)	5.98 (br. s)	5.92, 5.99 (d, 1)	5.96, 5.98 (br. s)
2⁗,6⁗				6.39 (s)	6.38 (4H, s)	6.39 (s)	6.34 (s)	6.34 (s)
OCH_3×2				3.81 (s)	3.81 (6H, s)	3.81 (s)	3.80 (s)	3.80 (s)
CH=				8.51(s)	8.51 (s), 8.53 (s)	8.51 (s)	8.57 (s)	8.57 (s)
NHC_6H_4–N=				—	—	—	7.97, 8.07 (d, 9)	7.97, 8.04 (d, 8)

[a]Proton could not be assigned due to complexity of the spectrum.

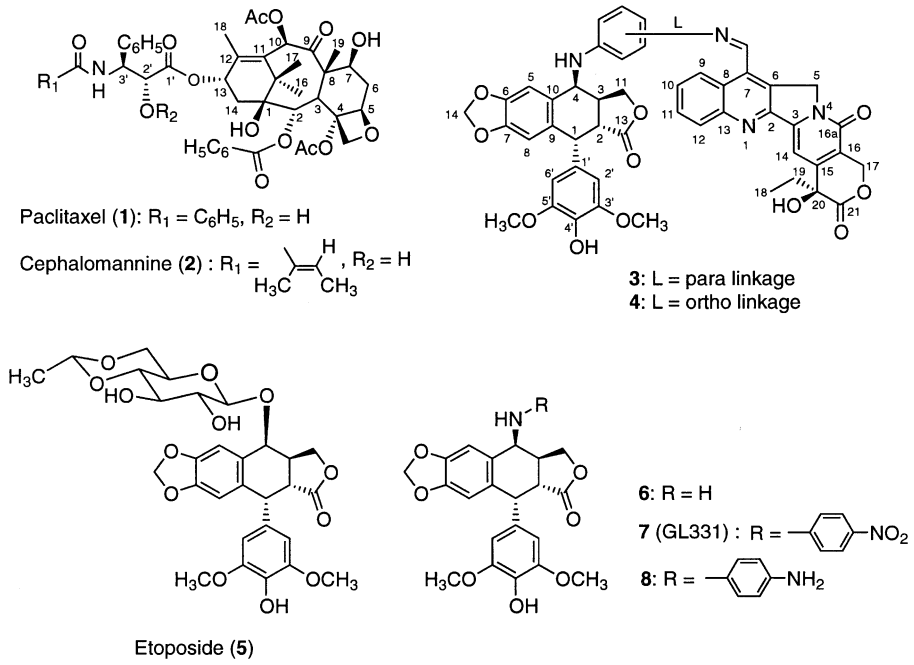

Figure 1. Structures of paclitaxel, cephalomannine, etoposide and its derivatives.

Table 2. Cytotoxicity of **12**–**16** in selected human tumor cell lines (ED_{50}) (μM)

Compound	KB	A549	HCT-8	CAKI-1	MCF-7	SK-MEL-2	1A9 (OVCAR)
12	0.003	0.21	0.54	>0.25	0.33	>0.40	0.03
13	16.5	NA[a]	NA	>4	>4	NA	ND
14	0.03	0.70	2.78	>5	1.23	8.90	0.14
15	0.23	2.80	>0.1	>4	1.86	>40	0.30
16	1.40	3.55	>40	>10	>5	>40	1.12
GL-331	1.86	0.48	3.38	1.80	9.29	5.95	0.20
Etoposide	0.20	1.95	>5	2.2	>5	ND	0.60
Cephalomannine	0.002	0.009	0.047	>0.3	0.167	0.134	0.003
Paclitaxel	0.016	0.006	0.013	ND[b]	ND	ND	0.002

[a]NA, not active.
[b]ND, not determined.

epipodophyllotoxins [etoposide (**5**) and GL-331 (**7**), see Fig. 1]. The HTCL panel was comprised of KB, A549, HCT-8, CAKI-1, MCF-7, SK-MEL-2, and IA9 cell lines. Two paclitaxel-resistant 1A9 sub-lines designated as PAX-10 and PAX-22 were also tested as part of the DRCL panel. Other resistant-cell lines were KB derivatives expressing various mechanisms or multi-drug resistance as described elsewhere.[14,15]

All conjugates except **13** showed significant cytotoxicity (data in Table 2). Although they were not as active as paclitaxel and cephalomanine, **12** and **14**–**16** displayed comparable or better activity than epipodophyllotoxin derivatives in most tumor cell lines. Interestingly, the cytotoxic profile of **15**, which is a conjugate of paclitaxel and **8**, was different from those of both precursors, suggesting that a novel mechanism of cell killing was achieved through conjugation. Compound **13**, which has an epipodophyllotoxin conjugated at both the taxoid 2- and 7-hydroxy groups, was relatively inactive. Thus, the taxoid 7-hydroxy group may be crucial for cytotoxic activity; a similar result was found previously by Mathew et al.[4] Compared with the unconjugated components, **12**, **15**, and **16** showed enhanced activity against paclitaxel-resistant cells (Table 3). Compounds **12**, **13**, and **15** were selected for further cytotoxicity evaluation in approximately 60 human tumor cell lines by NCI. The average log GI_{50} values over all cell lines for each tumor type are presented in Table 4. Compound **12** showed the strongest inhibitory effects against a variety of tumor cell lines, especially leukemia, colon, and prostate cell lines.

Table 3. Cytotoxicity of **12**–**16** in drug resistant cell lines (resistance fold)

Compound	KB-7d	KB-VCR	KB-CPT	PAX-10	PAX-22
12	1	17	1	7	17
13	0.6	—[a]	—	—	—
14	—	—	—	>36	—
15	1	8	1	>17	8
16	—	—	—	7.5	—
GL-331	2	3	1	13	8
Etoposide	119	153	1	>16	>16
Cephalomannine	—	—	—	40	—
Paclitaxel	1	15	1	24	24

[a]ND, not determined.

Table 4. Cytotoxicity of **12**, **13**, and **15** in NCI human tumor cell lines (log GI_{50})[a]

Compd	Leukemia	NSCL[b]	Colon	CNS[c]	Melanoma	Ovarian	Renal	Prostate	Breast
12	−7.60	−6.58	−7.20	−6.93	−6.29	−6.55	−6.13	−7.22	−6.56
13	−4.95	−5.78	−4.90	−5.20	−5.16	−5.03	−5.08	−5.06	−4.93
15	−6.02	−5.50	−5.12	−5.54	−5.01	−4.95	−5.88	−5.06	−4.96

[a]The data were provided by the NCI. GI_{50} is the concentration that caused 50% inhibition of tumor cell growth.
[b]Non-small cell lung cancer.
[c]Central nervous system cancer.

Table 5. Topoisomerase II inhibitory activities of **12**–**16**

Compound	Inhibition of Topo II IC_{100} (μM)	Cellular protein-DNA complex formation (%)
12	100	8±2
13	50	NA[a]
14	NA[b]	NA[b]
15	100	25±11
16	NA[b]	NA[b]
GL-331	50	134±7
Etoposide	100	100
Cephalomannine	NA[b]	NA[b]
Paclitaxel	NA[a]	NA[a]

[a]Not active at 50 μM.
[b]Not active at 100 μM.

In the topoisomerase assays, all three conjugates inhibited topo II in vitro but only **12** and **15** were intracellular poisons (Table 5). Compound **13**, which contains two epipodophyllotoxin moieties, did not stimulate protein-DNA breaks in cells. However, in the topo II inhibitory assay, **13** was twofold more active than **12** and **15** and comparable to GL-331, suggesting that a free hydroxy group might play a role in drug transport into tumor cells.

Experimental

Taxol and cephalomannine were kind gifts of Yung-Shin Pharm. Co., Taiwan. The proton nuclear magnetic resonance (^{1}H NMR) spectra were measured on a Bruker AC-300 spectrometer or Varian Inova 600 with Me_4Si (TMS) as the internal reference and $CDCl_3$ as solvent. Atmosphere Pressure Chemical Ionization (APCI) Mass was determined by PE Sciex API 150 Mass spectrometer. Thin-layer chromatography (TLC) silica gel plates were purchased from Alltech, Inc.

General procedure for synthesizing (4-formyl)benzoyl taxoids

To a solution of taxoid in anhydrous CH_2Cl_2 was added an equal molar ratio of 4-carboxylbenzaldehyde (2 equiv were used for **10**) and a catalytic (ca. 5%) amount of 4-dimethylaminopyridine (4-DMAP). To the mixture then was added an equal molar amount of 1,3-dicyclohexylcarbodiimide (DCC). The mixture was stirred under N_2 at room temperature for 24 h with TLC monitoring. The dicyclohexylurea precipitate was filtered and washed with CH_2Cl_2. The filtrate was concentrated to afford a white solid, which was further chromatographed over Si gel with an Elutflash® flashed silica gel column and eluted with CH_2Cl_2/MeOH = 10:0 to 20:1 to yield the desired **9**, as a white powder.

2-(4-Formyl)benzoyl paclitaxel (9). Yield 92% (starting with 11.7 g of taxol); amorphous; ^{1}H NMR δ ($CDCl_3$) see Table 1; APCI-MS m/z 986.6 ($M+H^+$).

2′,7-Bis(4-formyl)benzoyl paclitaxel (10). Same as general procedure except 1 mmol of paclitaxel and 2 mmol of 4-carboxylbenzaldehyde and DCC were used. Yield 95% (starting with 19.7 mg of paclitaxel). ^{1}H NMR δ ($CDCl_3$) see Table 1; APCI-MS m/z 1118.8 ($M+H^+$).

2′-(4-Formyl)benzoyl cephalomannine (11). Yield 95% (starting with 205 mg of cephalomannine); amorphous; ^{1}H NMR δ ($CDCl_3$) see Table 1; APCI-MS m/z 964.6 ($M+H^+$).

General procedure for synthesizing target compounds 12–16. To a solution of mono or bis 2′-(4-formyl)benzoyl taxoid (**9**–**11**) in anhydrous benzene was added an appropriate epipodophyllotoxin derivative (1 or 2 equiv **6** or **8**). The mixture was heated to reflux with a Dean Stark trap for 24 h. The solvent was distilled and the residue was purified by silica gel column chromatography eluting with CH_2Cl_2/MeOH = 20:1 to afford the desired compounds.

Conjugate 12. A yellow powder in 92% yield [starting with 22.5 mg (0.023 mmol) of **9** and 10.0 mg (0.025 mmol) of 4′-*O*-demethyl-4β-amino-4-desoxypodophyllotoxin (**6**)]; amorphous; ^{1}H NMR δ ($CDCl_3$) see Table 1; APCI-MS m/z 1367.7 ($M+H^+$).

Conjugate 13. A yellow powder in 89% yield [starting with 7.0 mg (0.006 mmol) of 2′,7-bis(4-formyl)benzoyl paclitaxed (**10**) and 5.1 mg (0.013 mmol) of **6**]; amorphous; ^{1}H NMR δ ($CDCl_3$) see Table 1; APCI-MS m/z 1864.7 ($M+H^+$).

Conjugate 14. A yellowish powder in 89% yield [starting with 39 mg (0.040 mmol) of 2′-(4-formyl)benzoyl Cephalomannine (**11**) and 16 mg (0.040 mmol) of **6**]; amorphous; ^{1}H NMR δ ($CDCl_3$) see Table 1; APCI-MS m/z 1345.8 ($M+H^+$).

Conjugate 15. A yellow solid in 70% yield [starting with 20.0 mg ((0.023 mmol) of compound **9** and 10.6 mg (0.025 mmol) of **8**]; amorphous; ^{1}H NMR δ ($CDCl_3$) see Table 1; APCI-MS m/z 1458.8 ($M+H^+$).

Conjugate 16. A yellow solid in 85% yield [starting with 39 mg (0.040 mmol) of compound **11** and 20 mg (0.040 mmol) of **8**]; amorphous; ^{1}H NMR δ ($CDCl_3$) see Table 1; APCI-MS *m*/*z* 1437.9 ($M+H^+$).

Biological assay

The in vitro cytotoxicity assay was carried out according to procedures described in Rubinstein et al.[16] Drug stock solutions were prepared in DMSO, and the final solvent concentration was $\leq$2% DMSO (v/v), a concentration without effect on cell replication. The human tumor cell line panel consisted of epidermoid carcinoma of the nasopharynx (KB), lung carcinoma (A-549), ileocecal carcinoma (HCT-8), renal cancer (CAKI-1), breast cancer (MCF-7), melanoma cancer (SKMEL-2), and ovarian cancer (1A9). The drug resistant cell line panel consisted of three KB-derivatives, -7d, -CPT, and -VCR. The properties of these cells are described elsewhere.[14,15] The PAX-10 and PAX-22 sub-line derivatives from 1A9 cells are resistant to paclitaxel and have mutations in β-tubulin.[17] Cells were cultured at 37 °C in RPMI-1640 with 100 μg/mL kanamycin and 10% (v/v) fetal bovine serum in a humidified atmosphere containing 5% CO_2. Initial seeding densities varied among the cell lines to ensure a final absorbance of 1–2.5 A_{562} units. Drug exposure was for 3 days, and the ED_{50} value, the drug concentration that reduced the absorbance by 50%, was interpolated from dose-response data. Each test was performed in triplicate, and absorbance reading varied no more than 5%.

Protein-linked DNA break and topoisomerase II activity assays

The procedures were done according to detailed published methods.[8]

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