Antitumor Agents 126.[1] Novel 4β-Substituted Anilino Derivatives of 3′,4′-*O*,*O*-Didemethylpodophyllotoxin as Potent Inhibitors of Human DNA Topoisomerase II

Zhe-Qing Wang,[2] Ya-Ching Shen,[2] Hong-Xing Chen,[3] Jang-Yang Chang,[3] Xin Guo,[3] Yung-Chi Cheng,[3] and Kuo-Hsiung Lee[2,4]

Received August 17, 1992; accepted September 1, 1992

A series of derivatives of 3′,4′*O*,*O*-didemethylpodophyllotoxin have been synthesized and evaluated for their inhibitor activity against neoplastic cell growth (KB) and against human DNA topoisomerase II as well as for their activity in causing cellular protein-linked DNA breakage. The results show that the compounds possessing a 4β-anilino moiety either unsubstituted or substituted at the para (F, $COOCH_3$, $COCH_3$, CN, CH_2CN, NO_2) or meta (OH) positions or with an ethylenedioxy moiety showed the same or greater activity than etoposide in causing cellular protein-linked DNA breakage and in inhibiting DNA topoisomerase II. However, compared to the corresponding 4′-*O*-demethyl analogues, the 3′,4′-*O*,*O*-didemethyl compounds have a similar potency in inhibition of DNA topoisomerase II but are less active in causing cellular protein-linked DNA breakage. Complete correlation between the three biological activities—cytotoxicity, inhibition of DNA topoisomerase II, and induction of protein-linked DNA breakage—was also not observed. This supports the possibility that the biological determinants of action among these compounds may be different.

KEY WORDS: etoposide; DNA topoisomerase II; anilino analogues of etoposide; KB cells; cytotoxicity.

INTRODUCTION

Two main hypotheses about the mechanisms of action of etoposide (1; VP-16), an anticancer drug (2–4), have been postulated. First, it interacts with DNA topoisomerase II (5–7), leading to DNA breaks. Second, oxidation of the 4′-phenolic hydroxyl group would lead to the formation of an orthoquinone (2) (8–18) and to the production of free radicals. Free radical-induced cleavage of DNA could then occur in the presence of Cu^{2+} and Fe^{3+} ions (19). The effect of 1 in causing DNA breaks requires the presence of a 4′-phenolic hydroxyl group, and thus masking of this group will, in general, lead to lower antitumor activity and enzyme interaction. The 3′-*O*-demethylation of 1 to the orthoquinone (2) by microsomal electron transport processes was first demonstrated by Sinha *et al.* (11). van Maanen *et al.* (16) have recently shown that 1 is oxidatively *O*-demethylated to catechol (3) by rat liver microsomes and purified rat liver microsomal cytochrome P-450. The catechol (3) was found to be capable of causing single- and double-stranded DNA breakage. Thus compounds 2 and 3 appear to be the active metabolites involved in causing DNA breakage without the participation of DNA topoisomerase II.

As an extension of our studies (20–28) aimed at the development of 4β-amino analogues of etoposide as potent inhibitors of human DNA topoisomerase II and as antitumor agents, we report herein on the synthesis and evaluation of new 4β-substituted anilino derivatives of 3′,4′-*O*,*O*-didemethylpodophyllotoxins. Previously, many 4β-substituted anilino derivatives of 4′-*O*-demethylpodophyllotoxin were found to be 2- to 10-fold more potent than 1 as inhibitors of topoisomerase II (25–27). Presumably, the 3′,4′-*O*,*O*-didemethyl-4β-substituted anilino analogues should be more efficient in causing free radical-induced DNA breakage, as they do not require metabolic activation. An evaluation of these catechol-bearing analogues as enzyme inhibitors and for their ability to cause DNA breakage is of current interest in elucidating the various cytotoxic mechanisms of 1 and related epipodophyllotoxins.

CHEMISTRY

The synthesis of the target compounds (11–25) was achieved using two different starting materials (4 and 8). As shown in Scheme I, podophyllotoxin (4) was oxidized to a 3′,4′-orthoquinone (5) using a modified method of Ayres and Lim (29). A lower temperature (5°C) and a very short reaction time (5 min) were used to prevent further oxidation. Catalytic hydrogenation of 5 afforded a catechol (6), which was then reacted with anhydrous HBr with inversion of the configuration of C-4 to yield the 4β-bromo (7) (30). The target compounds 11–25 were synthesized from 7 under SN1 conditions using substituted anilines as nucleophiles.

Whereas introduction of the arylamino group into the C-4 position of 4′-*O*-demethylpodophyllotoxin was stereoselective, giving rise to a predominant 4β product (26), reaction of substituted anilines with the 3′,4′-*O*,*O*-didemethyl derivative, 7, gave a mixture of 4β- and 4α-epimers in a range of 3:1 to 1:1, as shown by NMR analysis of the crude reaction product. The bulky α-oriented pendant aromatic ring E provides steric hindrance, blocking the nucleophilic attack of the anilines from the α-side. However, in the 3′,4′-*O*,*O*-didemethyl compounds, the assistance of hydrogen bonding between the 3′-OH and the amino group of the anilines as shown in Fig. 1 could result in more attack occurring from the α-side, yielding a higher proportion of the α-epimer.

The synthesis of 7 was also achieved by treatment of 10 with HBr. Compound 10 was obtained from 9 by catalytic hydrogenation, and 9 was prepared from 8 by oxidation with HNO_3 (Scheme I).

MATERIALS AND METHODS

General Experimental Procedures. All melting points were taken on a Fischer–Johns melting-point apparatus and are uncorrected. IR spectra were recorded on a Perkin–

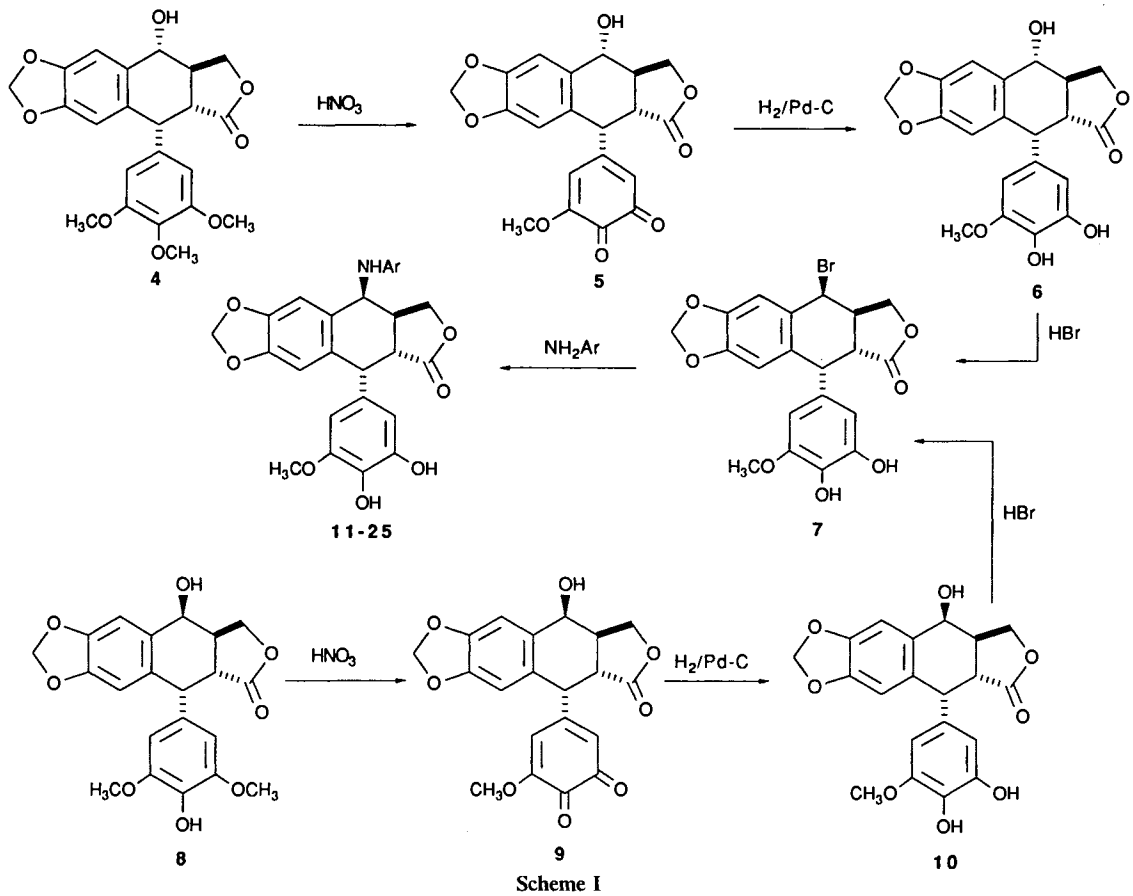
[1] For Part 125, see Ref. 1.

[2] Natural Products Laboratory, School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599.

[3] Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06520.

[4] To whom correspondence should be addressed.

0724-8741/93/0300-0343$07.00/0



Scheme I

Elmer 1320 spectrophotometer. ^{1}H NMR spectra were obtained on a Bruker AC-300 NMR spectrometer; all chemical shifts are reported as parts per million from TMS. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Optical rotations were measured with a Rudolph Research Autopol III polarimeter. Analytical thin-layer chromatography (TLC) was carried out on Merck precoated silica gel 60 F-254 plates. EM Kieselgel 60 (230- to 400-mesh ASTM) was used for column chromatography. All new target compounds were characterized by melting point, optical rotation, ^{1}H NMR and IR spectral analyses, and elemental analyses.

3',4'-O,O-Didemethyl-3',4'-dioxopodophyllotoxin (5). Compound 5 was prepared using a modified method of Ayres and Lim (29). A mixture of 90% nitric acid (3.0 ml) and glacial acetic acid (25 mL) precooled to 5°C was added in portions to a suspension of podophyllotoxin (4; 2 g, 4.8 mmol) in glacial acetic acid (25 mL) cooled at 5°C. The resulting deep red solution was stirred for 5 min and then poured into ice water (500 mL). The precipitate was extracted with chloroform (three times). After the combined organic layers were washed with brine until the pH approximated 5–6, it was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to yield the title compound (1.39 g, 75%) after recrystallization from toluene; mp, 190–192°C [literature (29) mp, 190–192°C].

3',4'-O,O-Didemethylpodophyllotoxin (6). A solution of 5 (2.0 g, 5.20 mmol) in methanol (300 mL) was stirred with 10% Pd/C (200 mg) under hydrogen at ambient pressure and temperature for 4 h. The catalyst was filtered off, and the filtrate was evaporated to yield a pale yellow solid, which was purified by column chromatography (silica gel 100 g with dichloromethane:acetone:methanol, 100:10:5, as an eluant) to give 1.9 g of 6. Yield, 95%; mp, 168–170°C; crystallized from ethyl acetate; $[\alpha]^{25}D$ − 75.6° (c = 0.5, CH_3COCH_3). IR (KBr) ν_{max}: 3420, 2900, 1752, 1700, 1610, and 1480 cm^{-1}. ^{1}H NMR (d_6-acetone) δ 7.41 (s, 1 H, 3'-OH), 7.39 (s, 1 H, 4'-OH), 7.17 (s, 1 H, 5-H), 6.52 (d, J = 1.7 Hz, 1 H, 6'H), 6.48 (s, 1 H, 8-H), 6.07 (d, J = 1.7 Hz, 1 H, 2'-H), 5.97 (s, 2 H, OCH_2O), 4.94 (d, 1 H, 4-OH), 4.76 (d, J = 9.4 Hz, 1 H, 4-H), 4.50 (t, J = 8.5 Hz, 1 H, 11-H), 4.49 (d, J = 4.5 Hz, 1 H, 1-H), 4.12 (t, J = 8.5 Hz, 1 H, 11-H), 3.74 (s, 3 H, 5'-OCH_3), 3.00 (dd, J = 14.3, 4.5 Hz, 1 H, 2-H), and 2.84 (m, 1 H, 3-H). *Anal.* ($C_{20}H_{18}O_8$). Calcd: C, 62.17; H, 4.70. Found: C, 62.09; H, 4.75.

3',4'-O,O-Didemethyl-4β-bromo-4-desoxypodophyllotoxin (7). A suspension of 6 (1.0 g, 2.59 mmol) in 25 mL of dry chloroform was kept at 0–5°C, and dry hydrogen bromide was passed through the suspension. After 30 min the bath was removed, and the solution was continuously stirred for 1 hr at room temperature. The solution was then evaporated, followed by using benzene as an azeotropic mixture to drive off the water formed in the reaction. The crude bromide 7 was used for the next reaction without purification. Compound 7 can also be prepared from 10 instead of from 6 by using the procedure described above.

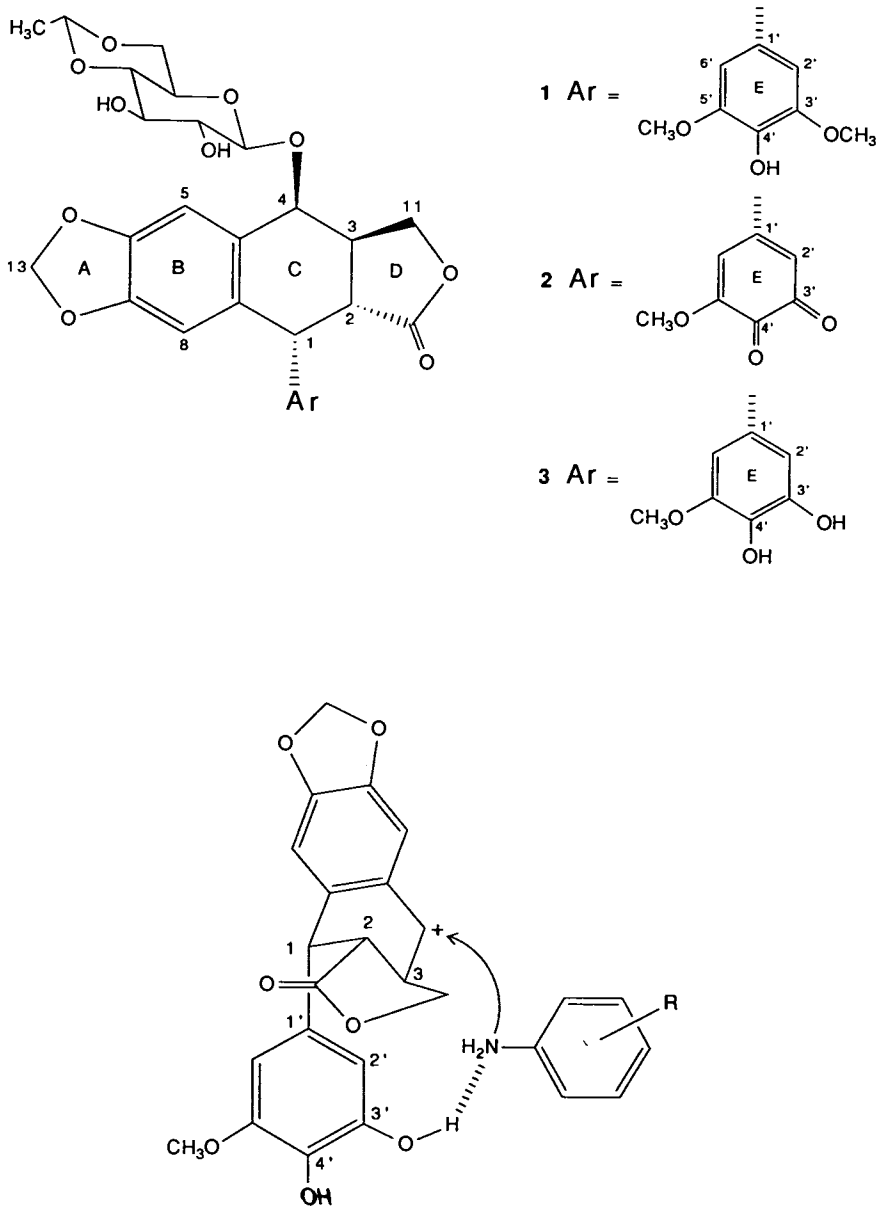


Fig. 1.

3′,4′-O,O-Didemethyl-3′,4′-dioxoepipodophyllotoxin (9). This compound (6.09 g) was synthesized from 8 (10 g, 24.3 mmol) using the aforementioned procedure for the preparation of 5 from 4. Yield, 65%; mp, 245–248°C; crystallized from toluene. IR (KBr) ν_{max}: 3480, 2920, 1768, 1695, 1660, 1626, 1560, and 1485 cm^{-1}. ^1H NMR ($CDCl_3$) δ 6.83 (s, 1 H, 5-H), 6.55 (s, 1 H, 8-H), 6.54 (s, 1 H, 6′-H), 6.02 (s, 2 H, OCH_2O), 5.21 (s, 1 H, 2′-H), 4.83 (d, J = 3.4 Hz, 1 H, 11-H), 4.52 (d, J = 1.3 Hz, 1 H, 4-H), 4.50 (d, J = 3.4 Hz, 1 H, 11-H), 4.30 (d, J = 5.6 Hz, 1 H, 1-H), 3.86 (s, 3 H, 5′-OCH_3), 3.50 (dd, J = 14.1, 5.6 Hz, 1 H, 2-H), and 2.83 (m, 1 H, 3-H). *Anal.* Calcd for $C_{20}H_{16}O_8$: C, 62.50; H, 4.20. Found: C, 62.33; H, 4.19.

3′,4′-O,O-Didemethylepipodophyllotoxin (10). This compound (5.1 g) was synthesized from 9 (5.18 g, 13.47 mmol) using the above-mentioned procedure for the preparation of 6 from 5. Yield, 98%; mp, 220–224°C; crystallized from methanol; $[\alpha]_D$ − 116° (c = 0.1, CH_3COCH_3). IR (KBr) ν_{max}: 3460, 2900, 1740, 1605, and 1470 cm^{-1}. ^1H NMR (d_6-acetone) δ 7.40 (d, 1 H, 3′-OH), 7.32 (s, 1 H, 4′-OH), 6.94 (s, 1 H, 5-H), 6.52 (s, 1 H, 6′-H), 6.47 (s, 1 H, 8-H), 5.99 (s, 2 H, OCH_2O), 5.95 (s, 1 H, 2′-H), 5.62 (s, 1 H, 4-OH), 4.89 (d, J = 3.1 Hz, 1 H, 4-H), 4.52 (d, J = 4.9 Hz, 1 H, 1-H), 4.32 (m, 2 H, 11-H), 3.73 (s, 3 H, 5′-OCH_3), 5.28 (dd, J = 14.1, 4.9 Hz, 1 H, 2-H), and 2.94 (m, 1 H, 3-H). *Anal.* Calcd for $C_{20}H_{18}O_8$: C, 62.17; H, 4.70. Found: C, 62.20; H, 4.65.

Synthesis of Compounds 11–25. A solution containing 7 (500 mg, 1.11 mmol), anhydrous barium carbonate (439 mg, 2.33 mmol), and the appropriate substituted aniline (1.16 mmol) in 8 mL of freshly distilled THF under nitrogen was stirred overnight at room temperature. The mixture was filtered and the filtrate was evaporated *in vacuo* to yield a

Table I. Physical Data for Compounds 11–25

Compound	Mp[a]	$[\alpha]^{25}_D$ (conc)[b]	Formula	Elemental analysis (C, H, N) Calc	Found	IR (KBr) τ max (cm^{-1})
11	170–173	−60° (0.1)	$C_{26}H_{23}NO_7$	67.67, 5.02, 3.04	67.57, 4.98, 3.15	3450, 3400, 1770, 1600, 1500, 1480
12	190–193	−78° (0.2)	$C_{26}H_{22}ClNO_7$	62.97, 4.47, 2.82	62.99, 4.43, 2.30	3450, 3390, 1760, 1590, 1495, 1475
13	175–178	−123° (0.3)	$C_{26}H_{22}FNO_7$	65.12, 4.62, 2.92	65.18, 4.59, 2.82	3450, 3400, 1765, 1610, 1500, 1475
14	206–209	−64° (0.07)	$C_{26}H_{23}NO_8$	65.40, 4.85, 2.93	65.51, 4.88, 2.88	3450, 3400, 1760, 1610, 1500, 1480
15	195–197	−94° (0.1)	$C_{26}H_{23}NO_8$	65.40, 4.85, 2.93	65.57, 4.80, 2.87	3500, 3200, 1755, 1605, 1500, 1472
16	155–158	−132° (0.1)	$C_{27}H_{25}NO_8$	65.98, 5.13, 2.84	65.68, 5.23, 2.82	3450, 3380, 1760, 1605, 1503, 1475
17	160–163	−101° (0.1)	$C_{28}H_{27}NO_9$	64.48, 5.21, 2.69	64.44, 5.25, 2.65	3450, 3390, 1760, 1605, 1505, 1475
18	181–183	−69° (0.2)	$C_{28}H_{25}NO_9$	64.73, 4.85, 2.70	64.66, 4.89, 2.81	3450, 3400, 1760, 1605, 1505, 1475
19	167–170	−84° (0.4)	$C_{28}H_{25}NO_9$	64.73, 4.85, 2.70	64.81, 4.83, 2.61	3450, 3390, 1770, 1600, 1510, 1480
20	178–180	−77° (0.2)	$C_{29}H_{27}NO_9$	65.28, 5.10, 2.63	64.99, 5.20, 2.72	3450, 3380, 1770, 1685, 1640, 1605, 1520, 1480
21	197–200	−114° (0.1)	$C_{27}H_{25}NO_8$	67.31, 5.22, 2.71	67.08, 5.31, 2.66	3450, 3350, 1760, 1640, 1590, 1510, 1475
22	210–212	−114° (0.1)	$C_{27}H_{22}N_2O_9$	61.94, 5.20, 5.35	61.89, 5.12, 5.47	3450, 3360, 2200, 1765, 1600, 1510, 1475
23	188–191	−85° (0.3)	$C_{28}H_{24}N_2O_9$	63.15, 4.54, 5.26	63.00, 4.41, 5.39	3450, 3380, 2240, 1760, 1605, 1505, 1475
24	182–185	−69° (0.1)	$C_{26}H_{22}N_2O_9$	61.66, 4.38, 5.53	61.43, 4.40, 5.49	3450, 3380, 1760, 1610, 1515, 1475
25	208–210	−105° (0.1)	$C_{26}H_{22}N_2O_9$	61.66, 4.38, 5.53	61.78, 4.22, 5.61	3450, 3380, 1760, 1590, 1490, 1470

[a] Recrystallized from Et_2O (°C).
[b] Optical rotations were run in acetone.

solid. This solid was chromatographed on a silica gel column (50 g) in toluene:ethyl acetate (3:1) and further purified by preparative TLC (Kieselgel 60 F-254. Art 5717, Merck), developed with toluene–ethyl acetate–methanol (9:3:0.2), to give compounds **11–25** with a yield which varied in a range of 20–30%.[5] The physical constants for the following compounds are given in Table I, and ^{1}H-NMR data are given in Table II: 3′,4′-*O*,*O*-didemethyl-4β-anilino-4-desoxypodophyllotoxin (**11**), 3′,4′-*O*,*O*-didemethyl-4β-(4″-chloroanilino)-4-desoxypodophyllotoxin (**12**), 3′,4′-*O*,*O*-didemethyl-4β-(4″-fluoroanilino)-4-desoxypodophyllotoxin (**13**), 3′,4′-*O*,*O*-didemethyl-4β-(3″-hydroxyanilino)-4-desoxypodophyllotoxin (**14**), 3′,4′-*O*,*O*-didemethyl-4β-(4″-hydroxyanilino)-4-desoxypodophyllotoxin (**15**), 3′,4′-*O*,*O*-didemethyl-4β-(4″-methoxyanilino)-4-desoxypodophyllotoxin (**16**), 3′,4′-*O*,*O*-didemethyl-4β-(3″4″-dimethoxyanilino)-4-desoxypodophyllotoxin (**17**), 3′,4′-*O*,*O*-didemethyl-4β-(3″,4″-ethylenedioxyanilino)-4-desoxypodophyllotoxin (**18**), 3′,4′-*O*,*O*-didemethyl-4β-(3″-methylcarboxyanilino)-4-desoxypodophyllotoxin (**19**), 3′,4′-*O*,*O*-didemethyl-4β-(4″-methylcarboxyanilino)-4-desoxypodophyllotoxin (**20**), 3′,4′-*O*,*O*-didemethyl-4β-(4″-acetylanilino)-4-desoxypodophyllotoxin (**21**), 3′,4′-*O*,*O*-didemethyl-4β-(4″-cyanoanilino)-4-desoxypodophyllotoxin (**22**), 3′,4′-*O*,*O*-didemethyl-4β-(4″-cyanomethylanilino)-4-desoxypodophyllotoxin (**23**), 3′,4′-*O*,*O*-didemethyl-4β-(3″-nitroanilino)-4-desoxypodophyllotoxin (**24**), and 3′,4′-*O*,*O*-didemethyl-4β-(4″-nitroanilino)-4-desoxypodophyllotoxin (**25**).

[5] The lower yields were due simply to the difficulty of separating the desired β-product from its α-isomer, which showed a nearly identical R_f value compared to that of the β-product on TLC.

RESULTS AND DISCUSSION

Table III shows the biological results for etoposide, **1**, and for new compounds **11–25** (A compounds) and their 4-*O*-demethyl analogues [B compounds (26)]. The third column gives a measure of the *in vitro* cytotoxicity, given as the ID_{50} for inhibition of KB cell growth. The fourth column shows the *in vitro* potency for inhibition of DNA topoisomerase II-dependent unknotting of P4 DNA. The final column gives a measure of cellular protein–DNA complex formation (%), which represents the intracellular inhibition of DNA topoisomerase II and the cellular protein-linked DNA breakage.

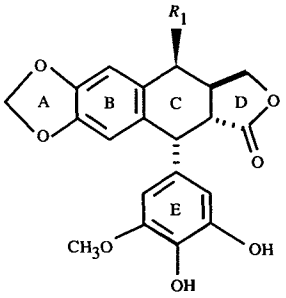
All of the new 3′,4′-*O*,*O*-didemethyl compounds (except for the *p*-Cl aniline derivative, **12**) showed equivalent or bet-

Table II. ^{1}H-NMR Spectra of 11–25[a]

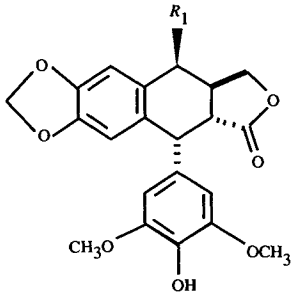
Compound	H-1	H-2	H-3	H-4	H-5	H-8	H_2-11	H_2-11	H-13	H-2′	OH-3′	OH-4′	Ome-5′	H-6′	NH	H-2″	H-3″	H-4″	H-5″	H-6″
11	4.54d	3.12dd	3.04m	4.64dd	6.73s	6.48s	4.35dd	3.97dd	5.92s[b]	5.84d			3.87s	6.47d	3.79d	6.51d	7.20t	6.76t	7.20t	6.51d
J[c]	4.8	13.9, 4.6		5.4, 4.4			8.6, 7.3	10.2, 8.6		1.8				1.8	6.0	7.8	7.8	7.8	7.8	7.8
12	4.53d	3.08dd	3.03m	4.57dd	6.70s	6.48s	4.33dd	3.92dd	5.93s[b]	5.81d	5.27br	5.27br	3.87s	6.74d	3.81d	6.44d	7.15d		7.15d	6.44d
J	4.4	13.9, 4.4		5.6, 4.2			8.6, 7.0	9.9, 8.6		1.9				1.9	5.7	8.8	8.8		8.8	8.8
13	4.52d	3.10dd	3.02m	4.54d	6.70s	6.47s	4.33dd	3.95dd	5.92s[b]	5.83d	5.27br	5.32br	3.86s	6.73d	3.70br	6.44dd	6.91dd		6.91dd	6.44dd
J	4.7	14.0, 4.9		4.0			8.2, 7.6	9.7, 8.2		1.5				1.5		8.9, 4.1	8.9, 8.6		8.9, 8.6	8.9, 4.1
14	4.53d	3.10dd	3.02m	4.61t	6.72s	6.48s	4.35dd	3.98dd	5.93br	5.84d	5.23br	5.31br	3.87s	6.72d	3.80d	6.02dd		6.10dd	7.03t	6.22dd
J	4.7	13.9, 4.7		5.5			8.4, 7.6	10.1, 8.4		1.6					5.5	2.0, 1.5		8.1,2.0	8.0	8.1, 2.0
15	4.52d	3.13dd	3.03m	4.52d	6.71s	6.47s	4.33dd	3.99dd	5.93br	5.83d	5.17br	5.28br	3.87s	6.77d		6.72d	6.41d		6.41d	6.72d
J	4.4	14.1, 5.0		4.4			8.3, 7.5	10.5, 8.3		1.6						8.6	8.7		8.7	8.6
16	4.52d	3.14dd	3.02m	4.54d	6.71s	6.47s	4.34dd	4.00dd	5.92br	5.83d	5.21br	5.30br	3.87s	6.73d		6.79d	6.46d	3.75s	6.46d	6.79d
J	5.0	14.0, 5.0		3.6			8.4, 7.7	10.7, 8.4		1.8				1.8		8.8	8.8	OCH_3	8.8	8.8
17	4.51d	3.12dd	3.01m	4.55d	6.73s	6.46s	4.32dd	3.99dd	5.93br	5.84d	5.36br	5.36br	3.85s	6.72d	3.61br	6.14d	3.81s	3.81s	6.74d	6.00dd
J	4.8	14.0, 4.8		3.7			8.6, 7.5	10.3, 8.6		1.2				1.2		2.7	OCH_3	OCH_3	8.6	8.6, 2.7
18	4.51d	3.12dd	3.00m	4.53d	6.71s	6.47s	4.35dd	4.00dd	5.92s[b]	5.85d	5.36br	5.36br	3.86s	6.70d	3.65br	6.05d	4.22m	4.22m	6.73d	6.03dd
J	4.8	14.0, 4.8		3.7			8.6, 7.5	10.4, 8.6		1.7				1.7		2.7	$-OCH_2$	CH_2O-	8.6	8.6, 2.7
19	4.54d	3.09br	3.09br	4.76dd	6.71s	6.49s	4.38dd	3.90dd	5.93s[b]	5.84d	5.16br	5.27br	3.87s	6.73d		7.18br	3.89s	7.44d	7.24t	6.70m
J	4.0			5.7, 3.5			6.8, 6.2			1.7				1.7			CO_2CH_3	7.6	7.7	
20	4.23d	3.06br	3.06br	4.54d	6.72s	6.49s	4.35dd	3.90dd	5.94s	5.82d	5.19br	5.32br	3.87s	6.73d	4.74br	6.51d	7.90d	3.85s	7.90d	6.51d
J	3.2			3.0						1.6				1.6		8.5	8.5	CO_2CH_3	8.5	8.5
21	4.51d	3.04br	3.04br	4.73br	6.68s	6.48s	4.33m	3.86dd	5.92s	5.83d	5.46br	5.46br	3.85s	6.70d	4.43d	6.52d	7.84d	2.49s	7.84d	6.52d
J	3.2									1.7				1.7	6.5	8.7	8.7	$COCH_3$	8.7	8.7
22	4.55d	3.06m	3.06m	4.30br	6.69s	6.50s	4.31dd	3.86dd	5.95s	5.80d	5.24s	5.29s	3.87s	6.74d	4.71m	6.53d	7.48d		7.48d	6.53d
J	4.0							10.9, 8.7		1.6				1.6		8.7	8.7		8.7	8.7
23	4.53d	3.10dd	3.04m	4.30dd	6.70s	6.48s	4.32dd	3.92dd	5.93br	5.83d	5.26br	5.26br	3.86s	6.72d	4.71m	7.14d	6.51d	3.64s	6.51d	7.14d
J	4.2	14.0, 4.2		4.9, 3.9			14.2, 7.3	9.8, 7.3		1.4				1.4		8.4	8.4	CH_2CN	8.4	8.4
24	4.56d	3.09br	3.09br	4.16d	6.70s	6.51s	4.40dd	3.90dd	5.95s	5.84d	5.17br	5.29br	3.87s	6.73d	4.72m	7.35m		7.61dd	7.35m	6.82dd
J	3.7			6.0						1.8				1.8				8.1,1.5		8.1, 2.4
25	4.56d	3.02dd	3.08m	4.56d	6.70s	6.51s	4.36dd	3.85dd	5.94s	5.80d	5.21br	5.32br	3.87s	6.73d	4.79m	6.53d	8.13d		8.13d	6.53d
J	4.7	14.3, 4.7					9.0, 7.1	11.2, 9.0		1.8				1.8		8.9	8.9		8.9	8.9

[a] Run in $CDCl_3$. Values are parts per million. s, singlet; d, doublet; t, triplet; m, multiplet; dd, doublet of doublets; br, broad.
[b] Two singlets separated by 0.01 ppm; total 2H for $-OCH_2O-$.
[c] Coupling constant (Hertz); when coupling constants are not given, peaks were overlapping.

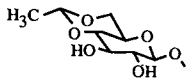
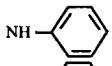
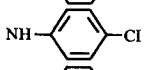
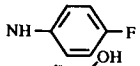
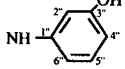
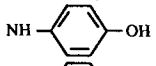
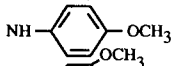
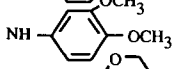
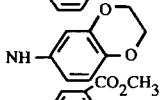
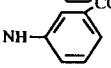
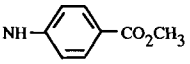
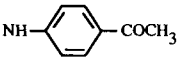
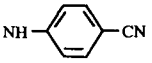
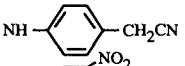
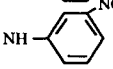
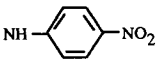
Table III. Biological Evaluation of 4β-(Substituted Anilino)-3′,4′-*O*,*O*-Didemethylpodophyllotoxins (11–25)



A



B

Compound	R_1	Cytotoxicity (ID_{50} KB: μ*M*)[a]		Inhibition of DNA topoisomerase II activity (ID_{50}; μ*M*)[b]		Cellular protein–DNA complex formation (%; 10 μ*M*)	
		A	B	A	B	A	B
1			0.2		50		100
11		1.8	0.7	25	25	128	243
12		2.0		>50		77	
13		1.5	0.2	10	5	117	213
14		1.7	0.5	25	25	105	290
15		1.7		25		83	
16		1.4		25		96	
17		1.0	<1.0	50	25	69	180
18		1.4	0.7	10	10	119	279
19		2.3	2.7	50	50	94	249
20		1.2	0.8	10	5	175	207
21		1.0		25		147	
22		1.8	0.6	10	10	146	211
23		1.2		25		109	
24		2.0	1.0	50	50	75	230
25		1.3	0.5	10	10	200	323

[a] ID_{50} is the concentration of drug which affords 50% of KB cell growth after 3-day incubation.

[b] Each compound was examined with five concentrations at 5, 10, 25, 50, and 100 μ*M*. The ID_{50} value was established based on the degree of inhibition at these five concentrations.

ter activity in inhibition of human DNA topoisomerase II (fourth column, A) compared to etoposide, **1**. Compounds **11**, **13–16**, **18–23**, and **25** were two- to fivefold more potent against this enzyme. In comparison to etoposide, **11**, **13**, **14**, **18**, **20–23**, and **25** also showed a greater percentage of cellular protein-linked DNA strand breakage (fifth column, A). All of the derivatives, however, showed a lower cytotoxicity toward KB cells (third column, A) than did etoposide.

As found previously with the 4′-*O*-demethyl compounds (26), complete correlation among the three activities with these new compounds is not observed. For example, one of the most cytotoxic catechol-containing compounds, **17**, is the least active in causing DNA breakage and in inhibition of topoisomerase II. In another example, compound **13** is fivefold more potent than etoposide in the topoisomerase inhibition assay but is only slightly more active in the protein–DNA breakage assay. Differences in metabolism and drug uptake could account for the differences found in the degrees of activity in the topoisomerase and protein–DNA complexation assays. Furthermore, the nature of the *in vitro* DNA topoisomerase assay is not as precise as the cellular protein–DNA complexation assay. The lack of correlation of the cytotoxicity and DNA breakage assays may suggest that the cytotoxicity of the 3′,4′-*O*,*O*-didemethyl compounds (**11–25**) may not by dependent solely on their inhibitory effects on DNA topoisomerase II, which results in causing protein-linked DNA breaks. Subsequent events which lead to the formation of double-stranded DNA breakage and cell death may also be affected by the nature of the compounds used.

When the 3′,4′-*O*,*O*-didemethyl compounds were compared to their 4′-*O*-demethyl analogues (A compounds to B compounds), similar activity was found in the topoisomerase II assay, while the catechol-containing compounds (A compounds) were less potent in causing protein-linked DNA breakage intracellularly. Also, the cytotoxicity toward KB cells was higher with the 4′-*O*-demethyl compounds (B compounds). It has been postulated that the cytotoxic action of these 4′-*O*-demethyl compounds could be mediated through the formation of free radicals. However, since the 3′,4′-*O*,*O*-didemethyl derivatives (A compounds) should form free radicals more easily but are also less cytotoxic, and there is a correlation of the cytotoxicity and protein-linked DNA breakage caused by a given pair of compounds, it is likely that the cytotoxic action of the 4′-*O*-demethyl compounds is still primarily a result of the interaction with DNA topoisomerase II.

BIOLOGICAL ASSAY

Assays for the inhibition of human DNA topoisomerase II and the cellular protein-linked DNA breaks as well as the cytotoxicity in KB cells were carried out according to the procedures described previously (21).

ACKNOWLEDGMENTS

The authors thank Mike Fisher of the Cancer Research Center, UNC—Chapel Hill, for KB cell culture assay. This work was supported by grants from the American Cancer Society, CH-370 and DHP-13E (K. H. Lee), and the National Cancer Institute, CA-44358 (Y. C. Cheng).

REFERENCES

1. X. M. Zhou, Z. Q. Wang, H. X. Chen, Y. C. Cheng, and K. H. Lee. Antitumor agents 125: New 4β-benzoylamino and 4β-benzoyl derivatives of 4′*O*-demethylpodophyllotoxin as potent inhibitors of human DNA topoisomerase II. *Pharm. Res.* 10:214–219 (1993).
2. I. Jardine, In J. M. Cassady and J. D. Douros (eds.), *Anticancer Agents Based on Natural Product Models*, Academic Press, New York, 1980, pp. 319–351.
3. B. F. Issell, F. M. Muggia, and S. K. Carter. *Etoposide [VP-16] Current Status and New Developments*, Academic Press, Orlando, FL, 1984, pp. 1–355.
4. H. Stahelin and A. von Wartburg. From podophyllotoxin glucoside to etoposide. In E. Jucker (Ed.), *Progress in Drug Research*, Birkhauser Verlag, 1989, Vol. 33, pp. 169–266.
5. W. Ross, T. Rowe, B. Glisson, J. Yalowich, and L. Liu. Role of topoisomerase II in mediating epipodophyllotoxin-induced DNA. *Cancer Res.* 44:5857–5860 (1984).
6. K. M. Tewey, G. L. Chen, E. M. Nelson, and L. F. Liu. Intercalative antitumor drugs interfere with the breakage-reunion reactions of mammalian DNA topoisomerase II. *J. Biol. Chem.* 259:9182–9187 (1984).
7. E. M. Nelson, K. M. Tewey, and L. F. Liu. Mechanism of antitumor drug action: Poisoning of mammalian DNA topoisomerase II on DNA by 4′-(9-acridinylamino)-methanesulfon-m-anisidide. *Proc. Natl. Acad. Sci. USA* 81:1361–1365 (1985).
8. J. D. Loike and S. B. Horwitz. Effect of VP-16-213 on the intracellular degradation of DNA in HeLa cells. *Biochemistry* 15:5443–5448 (1976).
9. A. H. Wozniak and W. E. Ross. DNA damage as a basis for 4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-*D*-glucopyranoside) (etoposide) cytotoxicity. *Cancer Res.* 43:120–124 (1983).
10. B. K. Sinha and C. E. Myers. Irreversible binding of etoposide (VP-16) to deoxyribonucleic acid and proteins. *Biochem. Pharmacol.* 33:3725–3728 (1984).
11. B. K. Sinha, M. A. Trush, and B. Kalyanaraman. Microsomal interactions and inhibitions of lipid peroxidation by etoposide (VP-16,213): Implications for mode of action. *Biochem. Pharmacol.* 34:2036–2040 (1985).
12. N. Haim, J. Roman, J. Nemec, and B. K. Sinha. Peroxidative free radical formation and *O*-demethylation of etoposide (VP-16) and teniposide (VM-26). *Biochem. Biophys. Res. Commun.* 135:215–220 (1986).
13. N. Haim, J. Nemec, J. Roman, and B. K. Sinha. *In vitro* metabolism of etoposide (VP-16-213) by liver microsomes and irreversible binding of reactive intermediates to microsomal proteins. *Biochem. Pharmacol.* 36:527–536 (1987).
14. J. M. S. van Maanen, E. Akker, J. D. Vries, T. R. Bakkenist, J. Lankelma, J. Retel, and H. M. Pinedo. Structure-bioactivation relationship of a series of podophyllotoxin derivatives. *Eur. J. Clin. Oncol.* 24:1415 (1988).
15. N. Haim, J. Nemec, J. Roman, and B. K. Sinha. Peroxidase-catalyzed metabolism of etoposide (VP-16-213) and covalent binding of reactive intermediates to cellular macromolecules. *Cancer Res.* 47:5835–5840 (1987).
16. J. M. S. van Maanen, J. D. Vries, D. Pappie, E. Akker, M. V. Lafleur, J. Retel, J. Greef, and H. M. Pinedo. Cytochrome P-450 medicated *O*-demethylation: A route in the metabolic activation of etoposide (VP-16-213). *Cancer Res.* 47:4658 (1987).
17. J. M. S. van Maanen, C. D. Ruiter, J. D. Vries, P. R. Koostra, F. Gobas, and H. M. Pinedo. The role of metabolic activation by cytochrome P-450 in covalent binding of VP-16-123 to rat liver and HeLa cell microsomal proteins. *Eur. J. Cancer Clin. Oncol.* 21:1099 (1985).
18. B. Kalyanaraman, J. Nemec, and B. K. Sinha. Characterization of free radicals produced during oxidation of etoposide (VP-16) and its catechol and quinone derivatives. An ESR study. *Biochemistry* 28:4839–4846 (1989).
19. H. Sakurai, T. Miki, Y. Imakura, M. Shibuya, and K. H. Lee. Metal- and photo-induced cleavage of DNA by podophyllotoxin, etoposide, and their related compounds. *Mol. Pharmacol.* 40:965 (1991).

20. L. Thurston, H. Irie, S. Tani, F. S. Han, Z. C. Liu, Y. C. Cheng, and K. H. Lee. Antitumor agents 78. Inhibition of human DNA topoisomerase II by podophyllotoxin and α-peltatin analogues. *J. Med. Chem.* 29:1547–1550 (1986).
21. S. A. Beers, Y. Imakura, H. J. Dai, Y. C. Cheng, and K. H. Lee. Antitumor agents 99. Synthetic ring C aromatized podophyllotoxin analogues as potential inhibitors of human DNA topoisomerase II. *J. Nat. Proc.* 51:901–905 (1988).
22. L. S. Thurston, Y. Imakura, M. Haruna, D. H. Li, Z. C. Kiu, S. Y. Liu, Y. C. Cheng, and K. H. Lee. Antitumor agents 100. Inhibition of human DNA topoisomerase II by cytotoxic ether and ester derivatives of podophyllotoxin and α-peltatin. *J. Med. Chem.* 32:604–608 (1989).
23. K. H. Lee, Y. Imakura, M. Haruna, S. A. Beers, L. S. Thurston, H. J. Dai, C. H. Chen, S. Y. Liu, and Y. C. Cheng. Antitumor agents 107. New cytotoxic 4-alkylamino analogues of 4′-demethylepipodophyllotoxin as inhibitors of human DNA topoisomerase II. *J. Nat. Prod.* 52:606–613 (1989).
24. S. Y. Liu, B. D. Hwang, M. Haruna, Y. Imakura, K. H. Lee, and Y. C. Cheng. Podophyllotoxin agents: Effects on DNA topoisomerase II, tubulin polymerization, human tumor KB Cells, and their VP-16-resistant variants. *Mol. Pharmacol.* 36:78–82 (1989).
25. K. H. Lee, S. A. Beers, M. More, Z. Q. Wang, Y. H. Kuo, L. Li, S. Y. Liu, J. Y. Chang, F. S. Han, and Y. C. Cheng. Antitumor agents 111. New 4-hydroxylated and 4-halogenated anilino derivatives of 4′-demethylepipodophyllotoxin as potent inhibitors of human DNA topoisomerase II. *J. Med. Chem.* 33:1364–1368 (1990).
26. Z. Q. Wang, Y. H. Kuo, D. Schnur, J. P. Bowen, S. Y. Liu, F. S. Han, J. Y. Chang, Y. C. Cheng, and K. H. Lee. Antitumor agents 113. New 4β-arylamino derivatives at 4′-*O*-demethylepipodophyllotoxin and related compounds as potent inhibitors of human DNA topoisomerase II. *J. Med. Chem.* 33:2660–2666 (1990).
27. J. Y. Chang, F. S. Han, S. Y. Liu, Z. Q. Wang, K. H. Lee, and Y. C. Cheng. Effect of 4β-arylamino derivatives of 4′-*O*-demethylepipodophyllotoxin on human DNA topoisomerase II, tubulin polymerization, KB cells, and their resistant variants. *Cancer Res.* 51:1755–1759 (1991).
28. Z. Q. Wang, H. Hu, H. X. Chen, Y. C. Cheng, and K. H. Lee. *J. Med. Chem.* 35:871–876 (1992).
29. D. C. Ayres and C. K. Lim. Modifications of the pendant ring of podophyllotoxin. *Cancer Chemother. Pharmacol.* 7:99–101 (1982).
30. M. Kuhn and A. von Wartburg. Synthesis and antimitotic activity of glycosidic lignan derivatives related to podophyllotoxin. *J. Med. Chem.* 14:936–940 (1971).