

NITROSOUREA DERIVATIVES OF 3',4'-DIDEMETHOXY-3',4'-DIOXO-4-DEOXYPODOPHYLLOTOXIN AND UREA DERIVATIVES OF 4'-Q-DEMETHYLPDOPHYLLOTOXIN AS POTENT INHIBITORS OF HUMAN DNA TOPOISOMERASE II^{1,†}

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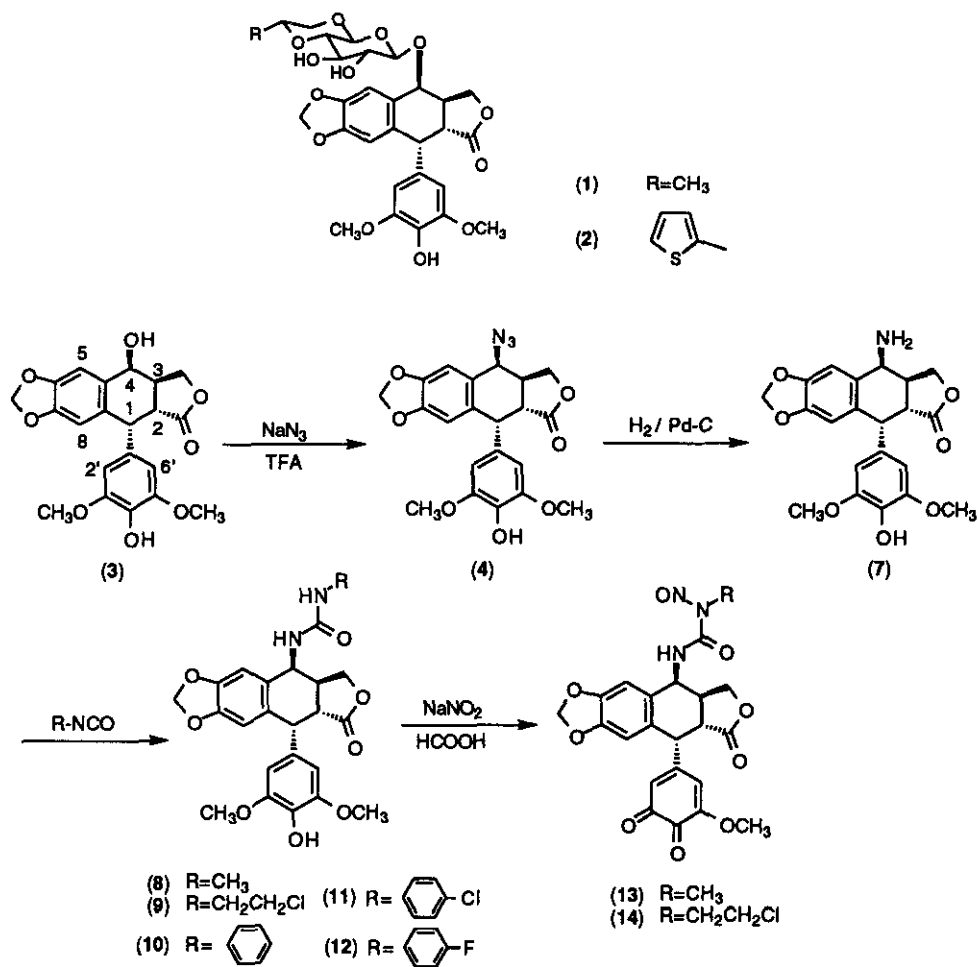
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Abstract — Nitrosourea derivatives of 3',4'-didemethoxy-3',4'-dioxo-4-deoxypodophyllotoxin and urea derivatives of 4'-Q-demethylpodophyllotoxin were synthesized and evaluated for their inhibitory activity against DNA topoisomerase II and KB cells. Although the 4 β -N'-nitrosoureido compounds demonstrated good inhibitory activity against topoisomerase II, they were found to possess low activity for protein linked-DNA complex formation ability. On the other hand, the 4 β -ureido compounds exhibited better or similar activity compared to 1, etoposide.

Etoposide (1; VP-16) and teniposide (2) are known to be potent chemotherapeutic agents for the treatment of small-cell lung carcinoma, testicular cancer, and malignant lymphoma. Since they inhibit the catalytic activity of DNA topoisomerase II by stabilizing a cleavable enzyme-DNA complex, in which DNA is cleaved and covalently linked to the enzyme, DNA topoisomerase II has been considered as a target enzyme of 1.² We

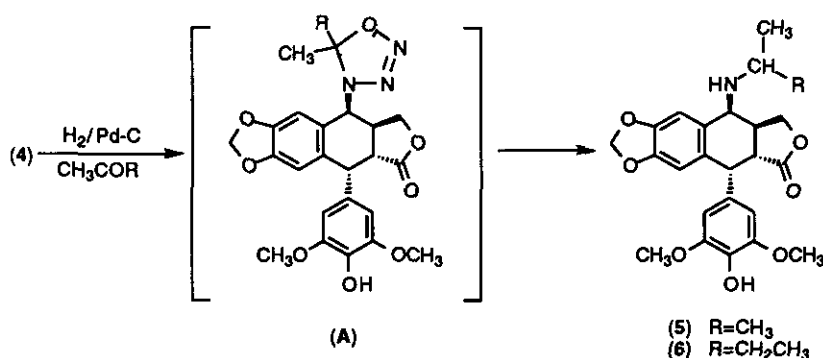
[†] Dedicated to Dr. Arnold Brossi on the occasion of his 70th birthday.

have previously synthesized and evaluated 4 β -aryl amino analogs related to 1, such as 4 β -aryl amino-3',4'-*Q,Q*-didemethylepipodophyllotoxins,³ -4'-*Q*-demethylepipodophyllotoxins,^{4,5} and -3',4'-dioxo-4-deoxypodophyllotoxins,⁶ as potent inhibitors of human DNA topoisomerase II and as antitumor agents. As an extension of our studies, we have synthesized new 4 β -substituted nitrosourea derivatives, together with 4 β -urea derivatives, aimed at developing agents for the treatment of brain tumors, since nitrosourea derivatives, such as *N,N*-bis-(2-chloroethyl)-*N*-nitrosourea (BCNU, Carmustine), *N*-(2-chloroethyl)-*N'*-cyclohexyl-*N*-nitrosourea (CCNU, Lomustine), and nitrosoureido sucroses, are able to penetrate the blood-brain barrier,⁷⁻⁹ and have been used clinically for the treatment of brain cancer.



Scheme 1

The nitrosourea derivatives were synthesized as shown in Scheme I. 4'-Q-Demethylepipodophyllotoxin (**3**) was treated with trifluoroacetic acid and NaN_3 to yield the 4 β -azido compound (**4**).⁵ When a ketone, such as acetone or methyl ethyl ketone, was used as the solvent, hydrogenation of **4** gave the corresponding 4 β -alkylamino derivatives (e.g., **5** and **6**). This could possibly occur through the cyclic intermediate (A) to yield the *N*-alkylaminopodophyllotoxin on reduction. Reduction of **4** in the absence of acetone or methyl ethyl ketone furnished the 4 β -amino compound (**7**). Compound (**7**) was further treated with the appropriate isocyanate to furnish the corresponding ureido derivatives (**8** – **12**).



In order to prepare the nitrosourea derivatives, **8** and **9** were treated with NaNO_2 in the presence of HCOOH . Unexpectedly, they yielded a red compound in each case (**13** and **14**, respectively). The ^1H -nmr spectrum of **13** exhibited signals due to H-1 [δ 4.45 (d, $J=4$ Hz)], H-2 [δ 3.26 (m)], H-3 [δ 3.68 (dd, $J=4, 14$ Hz)], H-4 [δ 5.46 (dd, $J=4, 8$ Hz)], H₂-11 [δ 4.23 (1H, dd, $J=2, 9$ Hz) and 4.55 (1H, dd, $J=1, 9$ Hz)], H-5 and -8 [δ 6.71 and 7.00 (each s)], and the methylenedioxy group [δ 6.00 (s)] of an epipodophyllotoxin skeleton. It also showed a three-proton singlet at δ 3.17, assignable to the *N'*-methylureido group, suggesting that the nitroso group was introduced at the distal nitrogen of the 4 β -*N'*-methylureido group. However, one of the two methoxy signals found in epipodophyllotoxin was absent [δ 3.76 (3H,s)], and the signals ascribable to H-2' and -6' were observed as a pair of doublets [δ 6.46 and 5.36 (each 1H, d, $J=1.5$ Hz)]. In addition, the observation of two carbonyl carbon signals (δ 175.0 and 179.1), along with an ester (δ 176.2) and ureido (δ 157.5) carbonyl signals, in the ^{13}C -nmr spectrum of **13**, suggested the presence of an *ortho*-benzoquinone ring. The ^1H -nmr of **14** was similar to that of **13**, except for the presence of the signals arising from the *N'*-nitroso-*N'*-2"-chloroethylureido group. These spectral data indicated that oxidation of ring-E had taken place,

together with nitrosation, giving the *ortho*-benzoquinone of the nitrosourea derivatives. The structures of the reaction products were concluded to be represented by 13 and 14. Since the ring-E of 4'-demethylpodophyllotoxins is quite sensitive to oxidating reagents, a nitrosourea derivative of 4'-demethylpodophyllotoxin could not be prepared, although several nitrosation conditions were attempted.

Compound (5) was also treated with NaNO_2 and HCOOH to afford the 4 β -*N*-nitroso-*N*-isopropyl derivative (15). Purification of 15 by silica gel chromatography (hexane-acetone) gave a yellow substance (16). The ^1H -nmr of 16 was similar to that of 13. A downfield shift of H-4 [δ 6.03 (1H, d, $J=4$ Hz)] compared with that [δ 3.92 (d, $J=4$ Hz)] of 5 indicated the existence of a *N*-nitroso group. An asymmetric signal pattern due to H-2' and -6' [δ 5.02 and 5.77 (each 1H, br s)] and a methoxy singlet [δ 3.82 (3H, s)], similar to those found in 13, suggested that again the ring-E was oxidized. However, an additional isolated methyl [δ 2.09 (3H, s)] and a methylene [δ 2.90 and 3.08 (each 1H, d, $J=15$ Hz)] in the ^1H -nmr spectrum, together with a carbonyl signal (δ 204.3) in the ^{13}C -nmr spectrum, suggested the presence of an acetonil group. In addition, the signals ascribable to C-4' and -5' were observed at δ 74.3 and 199.4 in the ^{13}C -nmr spectrum, suggesting that the acetonil group was attached at either C-4' or -5' through a carbon-to-carbon linkage. The location of the acetonil group was concluded to be at C-4' by ^1H - ^{13}C long-range COSY, which showed a long-range correlation between the methylene proton signal of the acetonil group and the C-3' carbon signal. Based on these spectral data, the structure of this reaction product was concluded to be represented by 16. The configuration of C-4' still remains to be determined. This acetonil group is considered to be derived from acetone, and similar examples are described in the literature.¹⁰

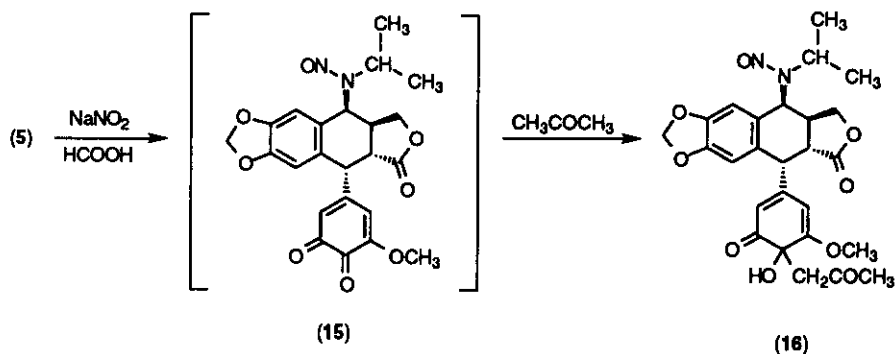


Table I shows the inhibition of DNA topoisomerase II activity, percentage of protein-linked DNA complex formation, and cytotoxicity against the KB cell line of the nitroso-urea and urea derivatives. The nitroso-urea derivatives (**13** and **14**) demonstrated good inhibitory activity against topoisomerase II. However, they possess low activity for protein linked-DNA complex formation ability. These results are similar to those found with 3',4'-didemethoxy-3',4'-dioxo-4-deoxy(epi)podophyllotoxin.⁶ In addition, the acetone condensed compound (**16**) showed no activity, although it does contain a *N*-nitroso group. This suggests that the *N*-nitroso group alone is not sufficient for inhibitory activity against topoisomerase II and that *ortho*-benzoquinone derivatives have decreased ability to cause protein-linked DNA complex formation. On the other hand, the *N*-ureido compounds (**8** – **12**) exhibited better or similar activity compared with etoposide, **1**. The aryl and β -chloroethyl-*N*-ureido compounds (**9** – **12**) also displayed cytotoxicity comparable to that of **1**.

Table I. Biological evaluation of nitroso-urea derivatives of 3',4'-didemethoxy-3',4'-dioxo-4-deoxypodophyllotoxin and urea derivatives of 4'-*Q*-demethylpodophyllotoxin

Compound	Cytotoxicity (ID ₅₀ KB, μ M) ^a	Inhibition of DNA topoisomerase II activity (ID ₅₀ , μ M) ^b	Cellular protein-DNA complex formation (% 20 μ M)
etoposide (1)	0.2	50	100
5	NT	50	109
6	NT	50	73
8	1.4	50	81
9	< 0.2	25	143
10	< 0.2	25	148
11	< 0.2	50	125
12	< 0.2	50	118
13	1.5	5	41
14	1.3	< 5	7
16	> 10	>100	1

^a ID₅₀ is the concentration of drug which affords 50% of KB cell growth after a 3 day incubation.

^b Each compound was examined with five concentrations at 5, 10, 25, 50 and 100 μ M.

The ID₅₀ value was established based on the degree of inhibition at these five concentrations.

NT : Not tested

EXPERIMENTAL

Optical rotations were determined using a Rudolph Research Autopol III polarimeter. ^1H and ^{13}C nmr spectra were recorded on a Bruker AC-300 (300 and 75.5 MHz, respectively) spectrometer. Chemical shifts are presented in terms of δ (ppm) with tetramethylsilane as an internal standard. Elemental analyses were performed by Atlantic Microlab, Inc. (Norcross, GA).

Synthesis of *N*-alkylamino Compounds. A solution of 4 β -azido-4'-demethyl-4-deoxypodophyllotoxin (**4**, 425 mg, 1 mmol) in a mixture of ethyl acetate (50 ml) and acetone or methyl ethyl ketone (20 ml) was treated with 10% Pd-C under H_2 atmosphere with stirring overnight. After filtration, the filtrate was concentrated to give a product, which was purified by silica gel chromatography [hexane-EtOAc (3 : 2)].

4 β -*N*-Isopropylamino-4-deoxy-4'-demethylpodophyllotoxin (5**).** Yield 75%; Colorless needles; mp 214 °C. $[\alpha]_{\text{D}}^{20} - 86.1^\circ$ ($c=0.55$, CHCl_3). Anal. Calcd for $\text{C}_{24}\text{H}_{27}\text{NO}_7 \cdot 1/2\text{H}_2\text{O}$: C, 63.99; H, 6.26; N, 3.11. Found: C, 64.36; H, 6.07; N, 3.28. ^1H -Nmr (CDCl_3 , 300 MHz): δ 6.79 (1H, s, H-5), 6.46 (1H, s, H-8), 6.29 (2H, s, H-2',6'), 5.96, 5.94 (each 1H, s, -O-CH₂-O-), 4.51 (1H, d, $J=5$ Hz, H-1), 4.26 (d, $J=9$ Hz, H-11), 3.92 (1H, d, $J=4$ Hz, H-4), 3.77 (6H, s, OCH₃), 3.26 (dd, $J=5, 14$ Hz, H-2), 2.83–2.65 (2H, m, H-3 and -1"), 1.21 and 1.05 (each 3H, d, $J=6$ Hz, 1"-CH₃).

4 β -*N*-Isobutylamino-4-deoxy-4'-demethylpodophyllotoxin (6**).** Yield 86%; Colorless needles; mp 220 °C. Anal. Calcd for $\text{C}_{25}\text{H}_{29}\text{NO}_7$: C, 65.92; H, 6.42; N, 3.08. Found: C, 65.64; H, 6.37; N, 3.04. ^1H -Nmr (CDCl_3 , 300 MHz): δ 6.79 (1H, s, H-5), 6.46 (1H, s, H-8), 6.29 (2H, s, H-2',6'), 5.96, 5.94 (each 1H, s, -O-CH₂-O-), 5.39 (1H, br s, NH), 4.51 (1H, d, $J=5$ Hz, H-1), 4.26 (2H, m, H-11), 3.97 (0.4H, d, $J=4$ Hz, H-4), 3.92 (0.6H, d, $J=4$ Hz, H-4), 3.77 (6H, s, OCH₃), 3.30 (3/5H, dd, $J=5, 14$ Hz, H-2), 3.23 (0.4H, dd, $J=5, 14$ Hz, H-2), 2.77 (1H, m, H-3), 2.61 (0.6H, m, H-1"), 2.45 (0.4H, m, H-1"), 1.70 (0.4H, m, H-2"), 1.38 (0.6H, m, H-2"), 1.28 (1.2H, d, $J=6$ Hz, 1"-CH₃), 1.01 (1.8H, d, $J=6$ Hz, 1"-CH₃), 0.93 (3H, t, $J=8$ Hz, 2"-CH₃). The ^1H -nmr examination showed that **6** is a mixture of diastereoisomers in a molar ration of 2 : 3.

Synthesis of 4 β -*N*-Ureido Compounds. A suspension of **7** (400 mg, 1 mmol) in benzene (50 ml) was added to a solution containing the appropriate isocyanate (0.20 mmol) in benzene (2 ml). The solution was stirred at room temperature overnight. The reaction mixture was concentrated to give a product, which was purified by crystallization (from hexane-EtOH) or silica gel chromatography [benzene-EtOAc (4 : 1)].

4 β -(*N*'-Methylureido)-4-deoxy-4'-O-demethylpodophyllotoxin (8**).** Yield 24%. Colorless crystals; mp 178 – 180 °C; $[\alpha]_{\text{D}}^{20} - 110.4^\circ$ ($c=0.5$, CHCl_3). Anal. Calcd for $\text{C}_{23}\text{H}_{24}\text{N}_2\text{O}_8 \cdot \text{H}_2\text{O}$: C, 58.22;

H, 5.52; N, 5.90. Found: C, 58.56; H, 5.46; N, 5.50. ^1H Nmr (CDCl_3 , 300 MHz): δ 6.82 (1H, s, H-5), 6.49 (1H, s, H-8), 6.27 (2H, s, H-2',6'), 5.96 and 5.93 (each 1H, s, -O-CH₂-O-), 5.44 (1H, br s, OH), 5.11 (1H, dd, $J=4$, 6.5 Hz, H-4), 4.66 (1H, d, $J=6.5$ Hz, NH), 4.51 (2H, d, $J=5$ Hz, H-1 and NH), 4.38 (1H, dd, $J=2$, 9 Hz, H-11), 3.96 (1H, t, $J=9$ Hz, H-11), 3.76 (6H, s, OCH₃), 2.85–2.90 (2H, m, H-2 and 3), 2.82 (3H, d, $J=5$ Hz, NHCH₃).

4 β -[N'-(2'''-Chloroethylureido)]-4-deoxy-4'-O-demethylpodophyllotoxin (9). Yield 24%. Colorless crystals; mp 225–230 °C; $[\alpha]_D^{20} - 160.0^\circ$ ($c=0.1$, CHCl_3). Anal. Calcd for $\text{C}_{24}\text{H}_{25}\text{N}_2\text{O}_8\text{Cl}\cdot\text{H}_2\text{O}$: C, 55.12; H, 5.20; N, 5.36. Found: C, 55.11; H, 5.15; N, 4.78. ^1H Nmr (CDCl_3 , 300 MHz): δ 6.82 (1H, s, H-5), 6.48 (1H, s, H-8), 6.26 (2H, s, H-2',6'), 5.95 and 5.91 (each 1H, s, -O-CH₂-O-), 5.45 (1H, br s, OH), 5.10 (1H, br s, NH), 5.10 (1H, d, $J=4$ Hz, H-4), 4.50 (1H, d, $J=4$ Hz, H-1), 4.38 (1H, t, $J=9$ Hz, H-11), 3.93 (1H, t, $J=9$ Hz, H-11), 3.75 (6H, s, OCH₃), 3.78–3.50 (4H, m, CH₂ $\times$ 2), 2.85–2.90 (2H, m, H-2 and 3).

4 β -[N'-(Phenylureido)]-4-deoxy-4'-O-demethylpodophyllotoxin (10). Yield 22%. Colorless crystals; mp 188–192 °C; $[\alpha]_D^{20} - 86.1^\circ$ ($c=0.55$, CHCl_3). Anal. Calcd for $\text{C}_{28}\text{H}_{26}\text{N}_2\text{O}_8\cdot 1/2\text{H}_2\text{O}$: C, 63.75; H, 5.16; N, 5.31. Found: C, 63.44; H, 5.38; N, 5.23. ^1H Nmr (CDCl_3 , 300 MHz): δ 7.00 (1H, br s, NH), 7.5–7.2 (5H in total, m, arom-H), 6.78 (1H, s, H-5), 6.40 (1H, s, H-8), 6.20 (2H, s, H-2',6'), 5.85 and 5.83 (each 1H, s, -O-CH₂-O-), 5.62 (1H, br s, OH), 5.08 (1H, d, $J=4$ Hz, H-4), 4.35 (1H, d, $J=4$ Hz, H-1), 4.25 (1H, t, $J=9$ Hz, H-11), 4.05 (1H, br s, NH), 3.86 (1H, t, $J=9$ Hz, H-11), 3.66 (6H, s, OCH₃), 2.68 (H, m, H-3), 2.65 (1H, dd, $J=4$, 14 Hz, H-2).

4 β -[N'-(4'''-Chlorophenyl)ureido]-4-deoxy-4'-O-demethylpodophyllotoxin (11). Yield 87%. Colorless crystals; mp 195 °C; $[\alpha]_D^{20} - 50.8^\circ$ ($c=0.69$, CHCl_3). Anal. Calcd for $\text{C}_{28}\text{H}_{25}\text{N}_2\text{O}_8\text{Cl}\cdot\text{H}_2\text{O}$: C, 58.90; H, 4.77; N, 4.91. Found: C, 59.13; H, 5.24; N, 5.37. ^1H Nmr (CDCl_3 , 300 MHz): δ 8.05 (1H, br s, NH), 7.52 and 7.26 (each 2H, d, $J=9$ Hz, arom.-H), 6.95 (1H, s, H-5), 6.53 (1H, s, H-8), 6.40 (2H, s, H-2',6'), 6.27 (1H, d, $J=7$ Hz, NH), 5.99 (2H, s, -O-CH₂-O-), 5.19 (1H, dd, $J=4$, 7 Hz, H-4), 4.56 (2H, d, $J=4$ Hz, H-1 and NH), 4.36 (1H, dd, $J=2$, 9 Hz, H-11), 4.04 (1H, t, $J=9$ Hz, H-11), 3.71 (6H, s, OCH₃), 3.1–3.0 (2H, m, H-2 and -3).

4 β -[N'-(4'''-Fluorophenyl)ureido]-4-deoxy-4'-O-demethylpodophyllotoxin (12). Yield 43%. Colorless crystals; mp 185 °C; $[\alpha]_D^{20} - 99.6^\circ$ ($c=0.6$, CHCl_3). Anal. Calcd for $\text{C}_{28}\text{H}_{25}\text{N}_2\text{O}_8\text{F}\cdot\text{H}_2\text{O}$: C, 60.65; H, 4.91; N, 5.05. Found: C, 60.13; H, 5.14; N, 4.62. ^1H Nmr (CDCl_3 , 300 MHz): δ 7.25 (2H, dd, $J=5$, 9 Hz, arom.-H), 7.00 (2H, t, $J=9$ Hz, arom.-H), 6.83 (1H, s, H-5), 6.77 (1H, br s, NH), 6.48

(1H, s, H-8), 6.25 (2H, s, H-2',6'), 5.94 and 5.92 (each 1H, s, -O-CH₂-O-), 5.15 (1H, dd, $J=4$, 7 Hz, H-4), 5.01 (1H, br s, NH), 4.48 (1H, d, $J=5$ Hz, H-1), 4.40 (1H, dd, $J=1$, 9 Hz, H-11), 3.97 (1H, t, $J=9$ Hz, H-11), 3.73 (6H, s, OCH₃), 2.95 (1H, m, H-3), 2.77 (1H, dd, $J=4$, 14 Hz, H-2).

Synthesis of *N*-Nitroso Compounds. To a solution of 4 β -*N*-ureido compounds (**5**, **8** or **9**, 1 mmol) in a mixture of chloroform (50 ml) and formic acid (200 mg), ground sodium nitrite (150 mg, 2.2 mmol) was added and was stirred for 1 h at 0 °C. The reaction mixture was washed with water, dried over sodium sulfate, and concentrated to give a residue, which was purified by silica gel chromatography [CHCl₃-EtOAc (9 : 1) or hexane-acetone (2 : 1)].

4 β -(*N*'-Nitroso-*N*'-methylureido)-3',4'-didemethoxy-3',4'-dioxo-4-deoxypodophyllo-toxin (13**).** Yield 40%. Brown powder; mp 175 °C (decomp.); $[\alpha]_D^{20} + 118.0^\circ$ ($c=0.1$, CHCl₃). Anal. Calcd for C₂₂H₁₉N₃O₉•1/2H₂O: C, 55.23; H, 4.21. Found: C, 55.67; H, 4.65. ¹H Nmr (CDCl₃, 300 MHz): δ 8.18 (1H, d, $J=8$ Hz, NH), 7.00 (1H, s, H-5), 6.71 (1H, s, H-8), 6.46 (1H, d, $J=1.5$ Hz, H-2'), 6.00 (2H, s, -O-CH₂-O-), 5.46 (1H, dd, $J=4$, 8 Hz, H-4), 5.36 (1H, d, $J=1.5$ Hz, H-6'), 4.45 (1H, d, $J=4$ Hz, H-1), 4.55 (1H, dd, $J=1$, 9 Hz, H-11), 4.23 (1H, dd, $J=2$, 9 Hz, H-11), 3.76 (3H, s, OCH₃), 3.68 (1H, dd, $J=4$, 14 Hz, H-2), 3.26 (1H, m, H-3), 3.17 (3H, d, $J=5$ Hz, NHCH₃).

4 β -(*N*'-Nitroso-*N*'-(2''chloroethylureido))-3',4'-didemethoxy-3',4'-dioxo-4-deoxypodophyllotoxin (14**).** Yield 33%. Brown powder; mp 200 °C (decomp.); $[\alpha]_D^{20} + 126.1^\circ$ ($c=0.1$, CHCl₃). Anal. Calcd for C₂₄H₂₅N₂O₈Cl•H₂O: C, 55.12; H, 5.20; N, 5.36. Found: C, 55.11; H, 5.15; N, 4.78. ¹H Nmr (CDCl₃, 300 MHz): δ 7.11 (1H, d, $J=8$ Hz, NH), 6.86 (1H, s, H-5), 6.48 (1H, s, H-8), 6.43 (1H, d, $J=1.5$ Hz, H-2'), 5.21 (1H, d, $J=1.5$ Hz, H-6'), 5.94 (2H, s, -O-CH₂-O-), 5.25 (1H, dd, $J=4$, 7 Hz, H-4), 4.24 (1H, d, $J=5$ Hz, H-1), 4.60 (1H, dd, $J=1$, 9 Hz, H-11), 4.14 (2H, t, $J=6$ Hz, H-2''), 4.10 (1H, t, $J=9$ Hz, H-11), 3.75 (3H, s, OCH₃), 3.49 (2H, t, $J=6$ Hz, H-1''), 3.15 (1H, dd, $J=2$, 14 Hz, H-2), 3.00 (1H, m, H-3).

4 β -(*N*-Nitroso-*N*-isopropyl)-3',4'-didemethoxy-3'-oxo-4'-hydroxy-4'-acetonil-4-deoxy-podophyllotoxin (16**).** Yield 33%. Yellow powder; mp 195 °C (decomp.); $[\alpha]_D^{20} - 56.9^\circ$ ($c=0.47$, CHCl₃). Anal. Calcd for C₂₆H₂₈N₂O₉•1/2H₂O: C, 59.87; H, 5.61; N, 5.37. Found: C, 60.28; H, 5.57; N, 5.33. ¹H Nmr (CDCl₃, 300 MHz): δ 6.65 (1H, s, H-5), 6.59 (1H, s, H-8), 6.04 (1H, d, $J=4$ Hz, H-4), 6.05, 6.01 (each 1H, s, -O-CH₂-O-), 5.77, 5.02 (each 1H, br s, H-2' and 6'), 4.50 (1H, dd, $J=1$, 9 Hz, H-11), 4.32 (1H, d, $J=4$ Hz, H-1), 3.82 (3H, s, OCH₃), 3.63 (1H, m, H-1''), 3.23 (1H, t, $J=9$ Hz, H-11),

3.20 (1H, m, H-3), 3.08, 2.90 (each 1H, s, $J=15$ Hz, acetonyl-CH₂), 2.78 (1H, dd, $J=5, 14$ Hz, H-2), 2.09 (3H, s, acetonyl-CH₃), 1.54, 1.53 (each 3H, d, $J=6.5$ Hz, 1"-CH₃).

Biological Assay. Assays for the inhibition of human DNA topoisomerase II, for the production of cellular protein-linked DNA breaks, and for the cytotoxicity towards KB cells were carried out according to the procedures described previously.¹¹

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