assays indicated that **4a** was converted to **1** in vivo. Compound **4a** (and by inference **4b**, **13**, and **13b**) may, therefore, be considered to be a prodrug of amiloride (**1**).

Experimental Section

Melting points were determined in open capillary tubes on a Thomas-Hoover Unimelt apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Varian EM 390 spectrometer, and ^{13}C NMR spectra were recorded on a Varian CFT-20 spectrometer. Chemical shifts are reported in δ values and ppm, respectively, relative to Me_4Si as internal standard. IR spectra were obtained on a Perkin-Elmer 297 spectrophotometer.

2-(3-Amino-1,2,4-oxadiazol-5-yl)-6-chloro-3,5-diaminopyrazine (4a). A suspension of 3,5-diamino-6-chloro-*N*-[(hydroxyimino)aminomethylene]pyrazine-2-carboxamide (23.82 g, 0.97 mol) in 200 mL of 2-propanol containing 2.3 g (0.1 mol) of sodium was refluxed on a steam bath for 12 h. The reaction mixture was cooled and filtered to give the crude product in quantitative yield. Recrystallization from DMF–H_2O afforded the analytical sample as a bright yellow solid: mp 278 °C; IR (KBr, partial) 3460, 3140, 1600, 1400, 1250, 1050, 880, 760 cm^{-1}; ^{1}H NMR (Me_2SO-d_6) δ 6.2 (2 H, br s), 7.35 (4 H, br s); ^{13}C NMR (Me_2SO-d_6) 107.27, 120.13, 152.73, 153.22, 167.70, 170.35 ppm. Anal. ($C_6H_6ClN_7O$) C, H, N.

N^{α}-Cyano-3-amino-5-(dimethylamino)-6-chloropyrazine-2-carboxamidine (10). To a solution of 800 mL of methanol containing 2.48 g (0.046 mol) of sodium methoxide was added 46.1 g (0.233 mol) of 3-amino-5-(dimethylamino)-6-chloropyrazinecarbonitrile in one portion. The resulting reaction mixture was stirred at room temperature for 30 h, filtered, and neutralized with 2.63 mL (0.046 mol) of acetic acid. The filtrate was concentrated to approximately 200 mL, treated with cyanamide (10.51 g, 0.25 mol), and allowed to stand at room temperature. After 5 h, the product (22.1 g) was collected and recrystallized from methanol to give the analytical sample as a bright yellow solid: mp 223–224 °C; IR (KBr, partial) 3400, 3150, 2190, 1600, 1550, 810, 775 cm^{-1}; ^{1}H NMR (Me_2SO-d_6) δ 3.15 (6 H, s), 7.5 (4 H, br s); ^{13}C NMR (Me_2SO-d_6) 40.54, 111.31, 115.76, 119.63, 152.61, 153.48, 165.11 ppm. Anal. ($C_8H_{10}ClN_7$) C, H, N.

2-(3-Amino-1,2,4-oxadiazol-5-yl)-3-amino-6-chloro-5-(dimethylamino)pyrazine (4b). To a solution of tetrahydrofuran (250 mL) containing 50 mL of methanol and 12.36 g (51.57 mmol) of **10** was added hydroxylamine hydrochloride (6.65 g, 103.13 mmol) and triethylamine (21.56 mL, 154.71 mmol). The resulting reaction mixture was protected from moisture and heated to reflux for 6 h. The reaction mixture was cooled, poured into water (1 L), and filtered. Concentration of the filtrate afforded more solid, which was combined with the initial precipitate to give 12.64 g (96%) of **4b** in analytically pure form: mp 210–211 °C; IR (KBr, partial) 1635, 1580, 1540, 1390, 1180, 910, 780 cm^{-1}; ^{1}H NMR (Me_2SO-d_6) δ 3.16 (6 H, s), 6.34 (2 H, s), 7.45 (2 H, s); ^{13}C NMR (Me_2SO-d_6) 40.6, 108.5, 120.7, 151.4, 153.3, 167.8, 170.1 ppm. Anal. ($C_8H_{10}ClN_7O$) C, H, N, Cl.

2-Methyl-3-amino-5-(6-chloro-3,5-diaminopyrazin-2-yl)-1,2,4-oxadiazolium Iodide (13a). The pyrazine–oxadiazole **4a** (2.03 g, 8.92 mmol) was dissolved in 20 mL of dry DMF with warming. The resulting solution was protected from moisture, treated with 10 mL of iodomethane, and allowed to stand at 40 °C overnight. The product, **13a**, was collected as a yellow solid in 94% yield: mp 211–213 °C dec; IR (KBr, partial) 3325, 3155, 1655, 1610, 1520, 1420, 1260 cm^{-1}; ^{1}H NMR (Me_2SO-d_6) δ 3.89 (3 H, s), 7.55 (2 H, br s), 8.10 (2 H, br s), 9.3 (2 H, br s); ^{13}C NMR (Me_2SO-d_6) 39.75, 102.94, 123.67, 154.13, 155.98, 161.82, 169.0 ppm. Anal. ($C_7H_9ClIN_7O\cdot{}^1/_4DMF$) C, H, N.

2-Methyl-3-amino-5-[3-amino-5-(dimethylamino)-6-chloropyrazin-2-yl]-1,2,4-oxadiazolium Iodide (13b). This compound was prepared in the same way as **13a** in 88% yield: mp 300 °C (from EtOH); IR (KBr, partial) 3075, 1670, 1520, 1465, 1395, 1180, 950 cm^{-1}; ^{1}H NMR (Me_2SO-d_6) δ 3.28 (6 H, s), 3.88 (3 H, s), 7.53 (2 H, br s), 9.3 (2 H, br s); ^{13}C NMR (Me_2SO-d_6) 36.43, 41.01, 103.51, 122.67, 153.56, 153.71, 161.59, 168.69 ppm. Anal. ($C_9H_{13}ClIN_7O$) C, H, N.

Acknowledgment. We acknowledge K. B. Streeter and associates for microanalyses, J. Murphy and Dr. D. W. Cochran for determining ^{13}C NMR spectra. We are indebted to H. Russo, Dr. K. C. Kwan, and their staffs for the biological data and to Dr. J. Springer and J. Hirshfield for the X-ray analysis. We thank Dr. S. J. de Solms, Dr. G. M. Smith, and O. W. Woltersdorf, Jr., for useful discussions and Drs. P. S. Anderson and R. F. Hirschmann for support and encouragement during the course of this investigation and M. Z. Banker for manuscript preparation.

Registry No. **4a**, 73631-29-5; **4b**, 80776-64-3; **8**, 32205-65-5; **9**, 14340-29-5; **10**, 80776-63-2; **13a**, 102652-56-2; **13b**, 80783-75-1.

Supplementary Material Available: Structure of **13b** and listings of fractional coordinates and temperature factors, bond distances, and bond angles of **13b** (4 pages). Ordering information is given on any current masthead page.

Pyrazole Derivatives. 5. Synthesis and Antineoplastic Activity of 3-(2-Chloroethyl)-3,4-dihydro-4-oxopyrazolo[5,1-*d*]-1,2,3,5-tetrazine-8-carboxamide and Related Compounds

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Two pyrazolotetrazine derivatives were synthesized as the analogous prodrugs of the light-sensitive antineoplastic agents dacarbazine and BIC. Both the pyrazole derivatives are stable under oridinary light illumination. Biological evaluation of these pyrazoles revealed that the compound containing a 2-chloroethyl function (**6a**) demonstrated good antineoplastic activity in experimental animals, but the one containing a methyl function (**6b**) was inactive. The inactivity of compound **6b** may suggest that compound **6a** and related imidazotetrazines may simply act as biological alkylating agents per se rather than as prodrugs. The information could also imply that the postulated dealkylation mechanism for the triazene derivatives should be reexamined.

The antineoplastic activity displayed by dacarbazine[1] [4-(3,3-dimethyl-1-triazeno)imidazole-5-carboxamide, DTIC, DIc, **1a**] in experimental animals[2] and in clinical trials[3] is well-known. It has been suggested that a meta-

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(1) Shealy, Y. F.; Krauth, C. A.; Montgomery, J. A. *J. Org. Chem.* **1962**, *27*, 2150.

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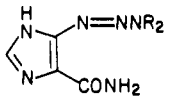
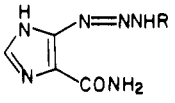
bolic intermediate, the N-demethylated homologue **2a**, is the active form of **1a**.[2c,4] However, this monomethyltriazeno derivative is not stable enough for practical use. In fact, both compounds **1a** and **2a** are light-sensitive, and the preparations of these compounds have to be carried out in the absence of light.[1,5,6]



1a, R = CH_3
b, R = CH_2CH_2Cl

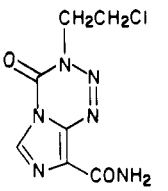


2a, R = CH_3
b, R = CH_2CH_2Cl

The corresponding nitrogen mustard derivative, 4-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-5-carboxamide (BIC, **1b**) has subsequently been synthesized and was found to have better antineoplastic activity than **1a**.[7] The mono(chloroethyl)triazeno compound **2b** has also been suggested as an active intermediate with the chloroethylcarbonium ion, $ClCH_2CH_2^+$, as the ultimate species in manifesting antineoplastic activity.[8] Unfortunately, compound **2b** is even less stable than the monomethyltriazeno compound **2a**. Again, both compounds **1b** and **2b** are sensitive and unstable under light.

To circumvent the problem of the instability of the active intermediate **2b**, a prodrug of **2b**, 3-(2-chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carboxamide (**3**), was ingeniously designed and synthesized by Stevens et al.,[9] utilizing the general synthetic method for azolotetrazinones of Ege and Gilbert.[10] Compound **3**, which was expected to undergo a ring-opening reaction in vivo to form the mono(chloroethyl)triazeno compound **2b**, demonstrated exceedingly high antineoplastic activity against leukemias L1210 and P388 in experimental animals.[9] However, the characteristic light sensitivity of the aforementioned imidazole derivatives is still retained with this prodrug, and its preparation also has to be conducted in the dark.[9]

In 1969, we found that the pyrazole analogues of dacarbazine, 3-(3,3-dimethyl-1-triazeno)pyrazole-4-carboxamide[11] (**4**), and the isomeric pyrazole derivative **5**[12] were



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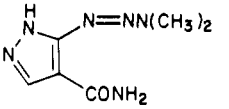

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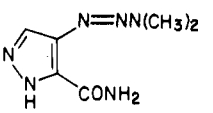

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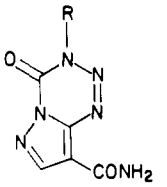
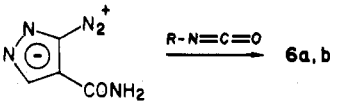
stable to both light and heat.[11,12] Preparation of these pyrazole derivatives can thus be carried out under the typical laboratory light illumination, and the finished products **4** and **5** can be stored in ordinary glass containers at room temperature for a long time. Compound **4** was found to possess antineoplastic activity comparable to dacarbazine by the National Cancer Institute (NCI) screen.[11]

We now report the synthesis of the novel pyrazole derivative **6a**, which has been demonstrated to be heat and light stable and highly active as an antitumor agent. Synthesis of the corresponding methyl analogue **6b** is also reported.



6a, R = CH_2CH_2Cl
b, R = CH_3

Chemistry. Compound **6** contains a pyrazolo[5,1-*d*]-1,2,3,5-tetrazine ring. This ring system was first reported by Ege and Gilbert[10] involving a [7 + 2] cycloaddition reaction of a diazopyrazole with an isocyanate. For the synthesis of **6a**, the required 3-diazopyrazole-4-carboxamide[13] (**7**) was originally prepared in our laboratory (the hazardous nature of **7** is described in the Experimental Section). Treatment of **7** with 2-chloroethyl isocyanate in EtOAc at room temperature, then heating at 50–60 °C, gave the desired **6a**. The compound was stable in $CHCl_3$ but was unstable in MeOH. In a similar manner, treatment of **7** with methyl isocyanate yielded the corresponding 3-methyl analogue **6b**.



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Compound **6a** was isolated from the reaction solution as a light-yellow crystalline solid, mp 193–194 °C. Although the product gave the correct elmental analysis and the expected parent peak as well as the fragmentation patterns from the mass spectrometry analysis, HPLC analysis of a $CHCl_3$ solution of the product revealed the presence of a small amount of impurity with an identical molecular ion of m/e 242. This impurity was assigned structure **8**, which was believed to be formed by the condensation of 4-hydroxypyrazolo[3,4-*d*]-*v*-triazine (**9**, readily formed from **7**[13]) and 2-chloroethyl isocyanate. Compound **8**, a urethane derivative, was found to be unstable in cold

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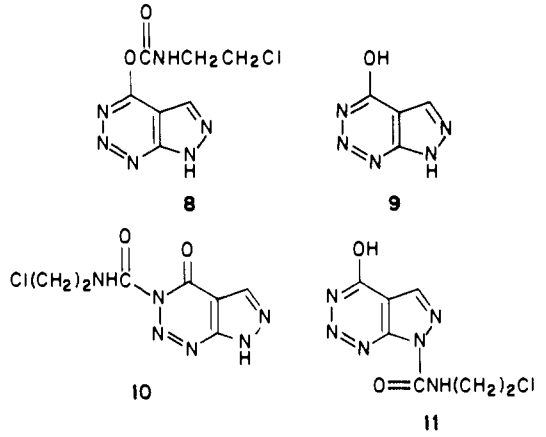
MeOH. This information suggested the structure of the impurity as 8 in preference to the isomeric urea derivatives 10 or 11. No additional work was conducted with this impurity material since it could be readily removed by column chromatography from the reaction product.



Biological Activity and Discussion. Screening information is provided in the Experimental Section.

Preliminary screening results from the National Cancer Institute for compound **6a** against leukemia P388 indicated that **6a** possessed outstanding inhibitory activity with T/C values at 164, 280 (8/12 mice survived more than 30 days), and 188 at doses of 120, 60, and 30 mg/kg, respectively. Compound **6b**, on the other hand, was totally inactive against the NCI P388 evaluation.

The Warner-Lambert in vivo screening gave the following results: Compound **6a** was highly active against L1210 leukemia (ip/ip; d 3–7; 40 mg/kg) and M5076 sarcoma (sc/ip; d 1–8; 34 mg/kg), producing over 90% cures (more than 60 days survival). It is also active against colon adenocarcinoma 11a (sc/ip; d 1–7; 23 mg/kg), producing a tumor growth delay of 18.3 days. Both **6a** and **6b** were inactive against mammary adenocarcinoma M16c.

It is rather surprising to note that while compound **6a**, which possesses a 3-(2-chloroethyl) substitution, exhibited outstanding antineoplastic activity against leukemia L1210 (comparable to that of the isomeric imidazole analogue, **3**), the corresponding 3-methyl analogue **6b** is inactive in antineoplastic evaluation. Earlier, it was suggested that N-demethylation of DTIC (**1a**) could be its major metabolic pathway[2c,4] and the resulting monomethyltriazeno derivative **2a** could be the active form of **1a**.[8] The same monodechloroethylation pathway was suggested for compound **1b** to **2b**. Consequently, compounds **6a** and **6b** were to be regarded as the prodrugs of **2b** and **2a**, respectively. (The antineoplastic activity of the methylpyrazole 4 against leukemia L1210 was reported.[11]) Notwithstanding, the present findings indicate that either compounds **6a** and **6b** do not undergo the proposed similar chemical transformation to the active species in vivo or the suggested metabolic pathways for **1a** and **1b** need to be reexamined so that proper active species could be unquestionably identified. Compound **6a** may be a biological alkylating agent per se.

Experimental Section

Melting points were determined on a Thomas-Hoover melting point apparatus. The ultraviolet spectra were recorded on a Varian Superscan 3 ultraviolet–visible spectrophotometer. The mass spectra were determined with an Atlas CH-5 mass spectrophotometer. Whereas analyses are indicated only by symbols of the elements, analytical results obtained by those elements were within ±0.4% of the theoretical values.

The National Cancer Institute in vivo screening was conducted as follows: Ascites fluid implanted in BDF_1 mice. Treatment schedule: qd 1–5.[14,15]

The Warner-Lambert in vivo screening was conducted as follows: Tumor lines were obtained either from the NCI Division of Cancer Treatment repository (L1210 and M5076) or from the Southern Research Institute (colon 11a and mammary 16c) and passaged according to standard techniques.[14,16,17] For testing, each tumor was implanted ip (10^4 cells, L1210 or trocar fragment, M5076, C11a, and M16c) in F_1 hybrid mice derived from the host of tumor origin and treated as indicated.[14,16,17] All test compounds were freshly dissolved for each injection in 10% aqueous Me_2SO and injected within 10 min of dissolution.

3-Diazopyrazole-4-carboxamide (3-Diazonium, Pyrazole-4-carboxamide, Hydroxide Inner Salt, 7). This compound was reported by us previously.[13] Following is a modified procedure that can be adapted *for larger scale synthesis.*

A stirred mixture of 18 g (0.103 mol) of finely powdered 3-amino-4-pyrazolecarboxamide hemisulfate[18] in 180 mL of H_2O was cooled at 0–5 °C. To this suspension was added, with vigorous stirring, a solution of 8 g (0.116 mol) of $NaNO_2$ in 10 mL of ice water. The mixture was stirred at 0–4 °C for 20 min and filtered through a sintered glass funnel. The wet solid was *thoroughly* washed with 2 × 20 mL of ice H_2O, 30 mL of 95% EtOH, 2 × 30 mL of absolute EtOH, and 2 × 40 mL of Et_2O. Agitation of the solid with a *porcelain* spatula during washing is very important in this operation. The remaining light-brown crystalline solid was then *carefully* transferred to a crystallizing dish and dried at room temperature in vacuo for 5 h to give 6.9 g (49% yield) of light-brown diazo compound, **7**. Its IR showed a characteristic triple-bond absorption peak at 2235 cm^{-1}. On slow heating in a melting point tube, the product decomposed gradually without melting, *but on rapid heating, the compound decomposed violently with a sharp sound at ca. 155–160 °C. Care, therefore, should be exercised for the handling of this compound. Rapid, repeated scratching of the dried powder with a metal spatula could cause a sudden decomposition of the product with an audible sound and a puff of smoke!*

From the filtrate there was obtained, on standing, 4.6 g (34% yield) of the triazine **9** as light golden shining platelets. This compound, on slow heating, did not melt below 300 °C and, on rapid heating, decomposed with a muffled sound at ca. 170 °C. Unlike compound **7**, its IR did not show the triple-bond absorption.

3-(2-Chloroethyl)-3,4-dihydro-4-oxopyrazolo[5,1-*d*]-1,2,3,5-tetrazine-8-carboxamide (Pyraoncozine, 6a). To a mechanically stirred suspension of 6.9 g (0.05 mol) of finely powdered **7** in 800 mL of dry EtOAc (CAUTION: do not powder the dry compound **7** alone; powder it with a glass rod in the EtOAc suspension) at room temperature was added 25 g (0.24 mol) of $Cl(CH_2)_2N{=}C{=}O$ followed by another 50 mL of dry EtOAc. The mixture was stirred at room temperature in the absence of moisture for 30 min, then heated at 50–55 °C with continuous stirring for 72 h. At the end of the period the suspension became a solution. A small amount of brown impurity was removed by filtration, and the filtrate was concentrated under reduced pressure at room temperature to 150 mL. The resulting light-yellow solid was collected by filtration, washed with 200 mL of Et_2O, and dried in vacuo at room temperature to give 3.2 g of pyraoncozine, **6a**: mp 188–189 °C with effervescence; UV λ_{max} ($CHCl_3$) 312 nm (ϵ 8500); mass spectrum, 242 (M^+), 226 ($M^+ - NH_2$), 193 ($M^+ - CH_2Cl$), 137 ($M^+ - ClCH_2CH_2N_3$), 94 ($M^+ - ClCH_2CH_2N_3$ –

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$CONH_2$ + H). Anal. ($C_7H_7ClN_6O_2 \cdot 0.5H_2O$) C, H, N.

A silica TLC plate (EtOAc) examination of the product revealed the presence of two minor spots in addition to the main spot (R_f 0.60). One of the minor spots did not move from the original place of application. It was found to be the residual pyrazolotriazine **9**. The other faint spot had an R_f value of 0.85 (compound **8**, formed by the condensation of **9** and $ClCH_2CH_2N{=}C{=}O$). Compound **8** has the same molecular weight and molecular formula as that of pyraoncozine, **6a**.

A slurry of 200 g of silica gel (60–200 mesh, J. T. Baker 2405-5) in EtOAc was packed into a 4.5 × 60 cm^2 column. To the wet-packed column was carefully added a well-mixed and finely ground solid powder coctaining 1.6 g of the crude pyraoncozine, **6a**, and 4 g of silica gel in EtOAc. The column was eluted with EtOAc. No TLC spots were detected in the initial 500 mL of EtOAc eluted. The TLC spot of the isomeric compound **8** was observed in the subsequent eluants; the fading of the undesired spot and the appearance of the desired one were monitored by continuous TLC examination from each of the eluted fractions. The fractions containing only the desired compound **6a** were collected (ca. 1500 mL) and evaporated to dryness. The resulting white fluffy crystals, mp 198–198.5 °C (eff), were recrystallized from 120 mL of EtOAc to give white fluffy needles, mp 200 °C (eff). The column purification was repeated and, from a total of 3.2 g of crude product, 1.6 g (13% yield) of chromatographically pure **6a** was obtained: UV λ_{max} ($CHCl_3$) 314 nm (ϵ 9000). Anal. ($C_7H_7ClN_6O_2$) C, H, N.

The entire operation was conducted under ordinary laboratory illumination. The product is stable under light even after 6 months. Pyraoncozine was found to be stable in $CHCl_3$ but unstable in MeOH. The ultraviolet absorption maximum of pyraoncozine in MeOH, on standing, gradually shifted from 314 to 265 nm.

Attempted use of less quantity of the isocyanate or less volume of EtOAc in subsequent trials resulted in less pure product.

3-Methyl-3,4-dihydro-4-oxopyrazolo[5,1-*d*]-1,2,3,5-tetrazine-8-carboxamide (6b). To a stirred suspension of 10 g (0.073 mol) of finely powdered **7** in 950 mL of EtOAc was added, at room temperature, 25 g (0.44 mol) of $CH_3N{=}C{=}O$. The suspension was stirred at room temperature for 4 days. To the resulting mixture (still a suspension) was added another 25 g (0.44 mol) of $CH_3N{=}C{=}O$, and the mixture was heated at 35–40 °C for an additional 4 days. The reaction mixture was filtered while hot, and the solid was washed repeatedly with EtOAc and dried. The light-brown solid product **6b** weighed 12 g (85% yield): mp 265–267 °C (dec). This compound was found to be much less soluble in EtOAc or $CHCl_3$ than the corresponding chloroethyl analogue: UV λ_{max} ($CHCl_3$) 310 nm (ϵ = 7200); mass spectrum, 194 (M^+), 137 ($M^+ - CH_3N_3$), 94 ($M^+ - CH_3N_3 - CONH_2$), 57 (CH_3N_3). Anal. ($C_6H_6N_6O_2$) C, H, N.

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Registry No. 6a, 90521-23-6; **6b**, 90521-24-7; **7**, 102613-59-2; **8**, 102587-14-4; **9**, 19818-50-9; $Cl(CH_2)_2NCO$, 1943-83-5; CH_3NCO, 624-83-9; 3-amino-4-pyrazole carboxamide hemisulfate, 102587-13-3.

Antitumor Agents. 78.[1] Inhibition of Human DNA Topoisomerase II by Podophyllotoxin and α-Peltatin Analogues

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It has been reported that the action of etoposide (VP-16) (**14**) as an antitumor agent is mediated through its interaction with DNA topoisomerase II which results in DNA breakage inside the cell. In order to understand the mechanism of action as well as structure–activity relationships of **14**, several novel, synthetic and some naturally occurring analogues related to podophyllotoxin were examined for inhibition of the DNA topoisomerase II activity. Compound **2** exhibited enhanced activity and compound **5** slightly diminished activity relative to **14**. A 4β-substituted ether at the C ring and O-demethylation at the E ring appear to enhance activity.

The class of 4′-demethylated epipodophyllotoxins, exemplified by clinical prototypes **14** and teniposide (VM-26), has emerged into the forefront of antitumor drug development. It is probable that type II DNA topoisomerase is required for the action of these drugs, which have been found to inhibit the DNA catenation activity of rat-derived enzyme.[2] The inhibition of this strand-passing function is likely the result of stabilization of the cleavable complex between DNA and type II DNA topoisomerase. These drugs produce single- and double-strand breaks in DNA in vitro in the presence of purified calf-thymus type II DNA topoisomerase,[19] as well as in vivo,[20] which are likely responsible for the cytotoxicity.[3] As part of our continuing investigation on the synthesis of novel potential antitumor podophyllotoxin analogues,[4] this paper describes the use of a rapid in vitro screening procedure for the evaluation of inhibition of the unknotting activity of human DNA topoisomerase II by these agents.

Chemistry

An adaptation of the method used by Kuhn et al.[5] was used to regioselectively O-demethylate the 4′-position of podophyllotoxin (**9**) and to invert stereochemistry of the C-4 hydroxyl group. The major product 4′-demethylepipodophyllotoxin (**1**) was accompanied by four other com-

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