whole was heated on a water bath for 20 min. After the mixture was cooled and water was added, the aqueous layer was extracted with ether. The ethereal extracts were washed with 1 N NaOH and water and then dried over Na_2SO_4, and the solvent was removed. The crude product was purified by column chromatography on silica gel (CH_2Cl_2 as the eluent). After recrystallization from EtOH, 12.7 g of **17** was obtained.

Method G. 1,1,2-Tris(4-methoxyphenyl)but-1-ene (29). A solution of **17** (3.74 g, 0.01 mol) in 250 mL of dry CH_2Cl_2 was cooled to −60 °C. Under nitrogen, BBr_3 (10.02 g, 0.04 mol) was added. After 0.5 h, the freezing mixture was removed, and the reaction mixture was stirred at room temperature for 4 h. After that, 50 mL of MeOH was added with cooling, and the solvents were removed under reduced pressure. Acetic anhydride (4.08 g, 0.04 mol) and pyridine (3.96 g, 0.05 mol) were added. The mixture was refluxed for 1 h. After the mixture was cooled, 300 mL of ice–water was added. The aqueous layer was extracted with ether, and the ethereal extracts were washed with 1 N HCl and saturated $NaHCO_3$ solution and dried over Na_2SO_4. The solvent was removed and the crude product was recrystallized from EtOH to give 3.48 g of **29**.

Biological Methods. Estradiol Receptor Binding Assay. The method described in ref 6 was used with some modifications. Test compounds were incubated with cytosol from calf uteri and [^{3}H]estradiol at 4 °C for 16 h. Incubation was stopped by adding dextran-coated charcoal. After centrifugation, the radioactivity of a 100-μL supernatant aliquot was counted. The percentage bound radioligand was plotted vs. the concentration of unlabeled test compounds. Six concentrations of the competitors were tested. They were chosen to provide a linear portion on a semilog plot crossing the point of 50% competition. From this plot, the molar concentrations of unlabeled estradiol and of test compounds reducing radioligand binding by 50% were determined.

Mammary Tumor Growth Inhibition Tests. (a) Hormone-Dependent Human Mammary Carcinoma Serially Transplanted in Nude Mice.[19] Animals of a random-bred strain (NMRI, nu/nu, castrated female or male mice, 10–12 animals per group) with a body weight of approximately 25 g and aged 6–7 weeks served as recipients. Tumor slices (0.5 cm in diameter) from postmenopausal women were transplanted under sterile conditions behind the shoulder blade into the area of the mammary gland of castrated male or female nude mice. The postmenopausal tumor was estrogen receptor and progesterone receptor positive. The receptors were measured by gel electrophoresis according to ref 28. Tumors were measured once a week by two diameters. Compounds were administered as olive oil solutions 6 times a week sc. Control animals received vehicle for a period of 4–6 weeks. At the beginning of treatment, tumor size was defined as "1".

(b) DMBA-Induced, Hormone-Dependent Mammary Carcinoma of the SD Rat. A single dose of 20 mg of DMBA (9,10-dimethylbenz[*a*]anthracene) was administered by gastric intubation to female SD (Sprague–Dawley) rats at an age of 50 days. After the appearance of tumors, about 4 weeks later, animals with at least one tumor with an area >140 mm^2 were classified in groups of ten. Compounds were administered in olive oil solution 6 times a week sc. The duration of treatment was 28 days. Measurement of tumor area was made twice weekly. The tumor area was defined by length × width of the tumor.

Acknowledgment. Thanks are due to the Deutsche Forschungsgemeinschaft and to the Verband der Chemischen Industrie, Fonds der Chemischen Industrie, who supported this work by grants. The technical assistance of A. Grobe and Mr. Stegmüller is gratefully acknowledged.

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Synthesis, 470-MHz ^{1}H NMR Spectra, and Activity of Delactonized Derivatives of the Anticancer Drug Etoposide[1]

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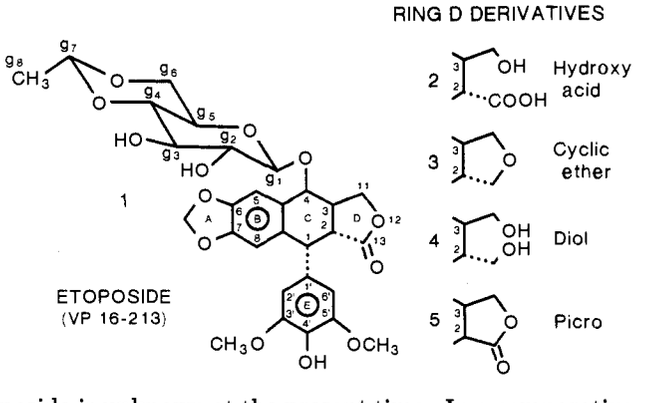
The anticancer drug etoposide (VP 16-213, **1**) contains a highly strained trans-fused γ-lactone. This functionality is readily metabolized to an inactive ring-opened hydroxy acid (**2**). To prevent this detoxification of the drug and to investigate whether the lactone is essential for drug activity, we synthesized a cyclic ether analogue of etoposide (**3**) and tested it in the mouse leukemia L1210 system in vitro and in vivo. This ether analogue of etoposide was found to retain activity in the L1210 system, but the activity was reduced relative to the parent drug. A synthetic intermediate, the ring D opened diol of the reduced lactone (**4**), was also tested and found to be inactive in the L1210 system. The complete 470-MHz ^{1}H NMR spectra of etoposide and its derivatives are reported. The usefulness of introducing deuterium at C-13 to determine $J_{2,3}$ is also demonstrated. This coupling constant is characteristic of cis or trans stereochemistry across the C–D ring fusion.

Etoposide (4′-demethylepipodophyllotoxin ethylidene-β-D-glucoside) (**1**) is a highly effective clinical anticancer agent.[2] The drug is a semisynthetic derivative of the potent antimitotic natural product podophyllotoxin.[3,4] Podophyllotoxin is the aglycon of etoposide with a methoxy group at C-4′ and the opposite configuration of the oxygen at C-4. However, whereas podophyllotoxin binds avidly to tubulin to prevent its polymerization, etoposide does not bind to tubulin and does not inhibit microtubule assembly. In fact, etoposide causes an irreversible blocking of the cell cycle in the late S and G_2 phases.[5] While effects on cellular respiration have been postulated to play a role in this regard,[6,7] the precise mechanism of action of etoposide is unknown at the present time. In a comparative study with other oncolytic agents, only X-irradiation



[†] Mayo Foundation.
[‡] Purdue University.

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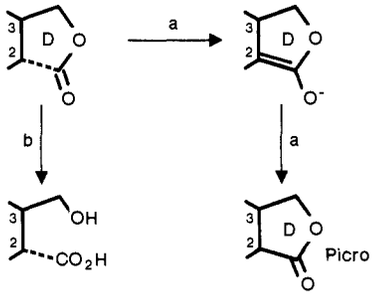
Scheme I



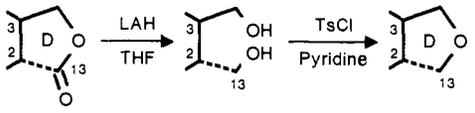
produced effects similar to etoposide.[8] Thus, this drug appears to be the first effective clinical agent of a new class of antitumor agents.

Podophyllotoxins, including etoposide, possess a highly strained trans-fused γ-lactone that can easily be epimerized in base to the thermodynamically favored cis lactone of the "picro" series of compounds (Scheme I, path a, partial structures shown) (e.g., **5**). The picro epimers of podophyllotoxins are generally much less active than the parent compounds in biological assays.[4] To prevent possible metabolic detoxification of podophyllotoxins through epimerization, Gensler et al.[9] synthesized a number of delactonized derivatives that could not enolize, including the corresponding cyclic ether in which the lactone carbonyl was converted to a methylene group. Gensler found that some of these derivatives retained considerable activity in several biological assays, although activity was always less than that of the parent compound.[9]

Etoposide is not metabolized by epimerization to its picro analogue[10] but instead is extensively deactivated to the ring-opened hydroxy acid[10,11] (**2**) (Scheme I, path b).

Synthesis of a derivative of etoposide which cannot be so readily inactivated via metabolism could potentially provide a better therapeutic agent whose pharmacokinetics[12-14] would be more consistent among patients. As evidenced by the chemical reactions of this lactone moiety[15,16] and by the ease of conversion of etoposide to the hydroxy acid in vivo[10,11] the carbonyl group is readily susceptible to nucleophilic attack. Such a reaction could lead to acylation of cellular macromolecules, and indeed a small percentage of teniposide (VM 26), a close analogue

Scheme II



of etoposide, has been demonstrated to bind irreversibly to the nuclear fraction of L1210 cells in vitro.[17] This nuclear binding, the nature of which has not been investigated, might play a role in the overall mechanism of action of the drug. To prevent metabolic inactivation by hydrolysis and to investigate the possible role of the lactone carbonyl relative to the drug's activity, we converted the D ring of etoposide to the analogous trans-fused cyclic ether (**3**) by changing the carbonyl to a methylene group. The activity of this new compound was measured in the mouse leukemia L1210 system in vivo and in vitro. The parent compound, etoposide and a synthetic intermediate, the opened ring D diol (**4**), were also tested.

The structures of these compounds were confirmed by extensive use of 470-MHz proton NMR and high-resolution mass spectrometry. Recent 360-MHz proton NMR observations of Brewer et al.[18] on etoposide have been extended by assigning resonances for the glycosidic protons. The resonances for the synthetic derivatives were also totally assigned.

Results and Discussion

The cyclic ether analogue of etoposide (**3**) was synthesized by reduction of etoposide with lithium aluminum hydride (LAH) in tetrahydrofuran (THF), followed by closure of the resulting diol (**4**) to a cyclic ether by *p*-toluenesulfonyl chloride (TsCl) in pyridine (Scheme II, partial structure shown). This procedure is similar to that of Gensler et al.[9] for the synthesis of the corresponding ether analogue of podophyllotoxin. The cyclization reaction went to completion using only 1 equiv of TsCl. The reaction was made as selective as possible for the primary hydroxy groups by using a lower temperature.[19] This selectivity was verified in that etoposide, reacting under the same conditions, yielded only starting material. It was surprising that the phenolic hydroxy did not tosylate, but this may be because of steric hindrance by the flanking methoxyl groups.

The structures of the diol (**4**) and the ether (**3**) were confirmed by extensive use of 470-MHz proton NMR (see Tables I and II). Total assignments of the proton NMR spectra were made possible by the use of homonuclear decoupling, together with the high resolution of 470-MHz proton NMR.

The opening of the lactone ring D to a diol, producing a conformationally less rigid ABC ring system, is evidenced by changes in the proton spin systems. In etoposide, the resonances for H-11 and H-11″ at δ 4.40 and 4.21 are well resolved at 470 MHz as distinct double doublets, showing the rigidity of the ABCD ring system. In **4** these diastereotopic protons exist in an ABX spin system, showing the comparative geometric freedom of the opened ring. Also, the H-13 and H-13″ protons, introduced in the reduction of etoposide, exist in an ABX spin system. Upon conversion of the relatively flexible diol (**4**) to the rigid ether (**3**), the protons at C-11 and C-13 are each found as distinct

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Table I. Chemical Shifts for Protons of Etoposide (1) and Its Derivatives[a]

proton position[b,c]	chem shift, ppm			
	1	5	4	3
5	6.81 (s)	6.77 (s)	6.74 (s)	6.77 (s)
8	6.54 (s)	6.39 (s)	6.40 (s)	6.49 (s)
2', 6'	6.25 (s)	6.44 (s)	6.18 (s)	5.99 (s)
3', 5'	3.75 (s)	3.86 (s)	3.76 (s)	3.75 (s)
OCH_2O	5.98 (d, d)	5.95 (d,d)	5.91 (AB)	5.95 (d,d)
4''	4.90 (d)	4.93 (d)	5.01 (d)	4.90 (d)
1	4.59 (d)	4.21 (d)	4.22 (d)	4.27 (d)
11''	4.40 (dd)	4.53 (ABX)	3.81 (ABX)	3.94 (dd)
11	4.21 (t)	4.48		3.82 (t)
2	3.24 (dd)	3.18 (dd)	2.62 (m)	2.81 (m)
3	2.86 (m)	2.96 (m)	2.26 (m)	2.44 (m)
13''			3.57 (ABX)	3.92 (dd)
13			3.49	2.93 (dd)
g1	4.65 (d)	3.92 (d)	4.65 (d)	4.56 (d)
g2	3.43 (t)	3.44 (t)	3.36 (m)	3.41 (dd)
g3	3.74 (t)	3.58 (t)	3.71 (t)	3.71 (t)
g4	3.34 (m)	3.32 (t)	3.36 (m)	3.32 (m)
g5	3.34 (m)	3.18 (m)	3.36 (m)	3.32 (m)
g6 ax.	3.56 (t)	3.58 (t)	3.60 (t)	3.58 (t)
g6 eq	4.16 (m)	4.15 (dd)	4.20 (dd)	4.18 (m)
g7	4.74 (q)	4.72 (q)	4.73 (q)	4.73 (q)
g8	1.39 (d)	1.35 (d)	1.38 (d)	1.37 (d)
g2-OH	2.44 (br s)	2.61 (br s)		
g3-OH	2.70	2.81		

[a] Abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; dd, double doublet; m, multiplet; d,d, distinct doublets; br s = broad singlet. [b] The positions of α and β protons at C-11 and C-13 were assigned by comparison to Brewer's data[18] for podophyllotoxin and podophyllotoxin cyclic ether and may be reversed. [c] In very dry $CDCl_3$ a slight splitting of the resonances for g2-OH and g3-OH were noted, which allowed assignment by selective decoupling experiments.

Table II. Coupling Constants for Protons of Etoposide (1) and Its Derivatives

proton position	*J*, Hz			
	1	5	4	3
1, 2	5.1	4.8	6.0	6.4
2, 3	14.0	9.4	12.0[a]	13.0[a]
3, 4''	3.4	3.0	3.0	3.2
3, 11	8.0	7.0		8.2
3, 11''	10.5	1.5		9.7
11, 11''	9.0	9.4	12.0	8.2
2, 13			5.5	10.9
2, 13''			6.5	7.4
13, 13''			11.0	7.4
OCH_2O	1.0	1.0	1.0	1.0
g1, g2	7.6	7.7	7.6	7.7
g2, g3	8.0	9.0	9.0	9.0
g3, g4		9.0	9.0	9.0
g4, g5				9.0
g5, g6 eq	4.3	4.5	4.4	4.9
g5, g6 ax.	9.5	9.0	10.0	9.0
g6 eq, g6 ax.	10.4	10.7	10.5	10.4
g7, g8	5.0	5.0	5.0	5.0

[a] Determined by irradiation of H-1 in the corresponding compounds dideuterated at C-13.

double doublet or triplet resonances. Again, this is indicative of the rigidity of the ring system and the distinct chemical environment of these diastereotopic protons.

Ayers and Pauwels[20] reported that the lactone of podophyllotoxin was reduced by LAH in THF to the corresponding diol with retention of stereochemistry at C-2. In view of the smooth epimerization of these lactones to the cis lactones of the picro series of compounds under mildly

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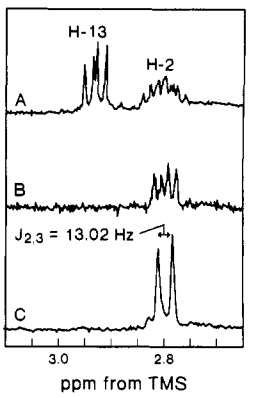

Figure 1. Sections of the 470-MHz ^1H NMR spectra of the nondeuterated (A) and deuterated (B, C) ether analogue (**3**) of etoposide. In A are shown the resonances for the H-2 proton and one of the H-13 protons. In B, the H-13 proton resonance has disappeared and the H-2 pattern has simplified after substitution with deuterium at C-13. In C, the H-1 proton has been decoupled from H-2 by irradiation at δ 4.25–4.30, allowing direct measurement of $J_{2,3}$ as 13.02 Hz.

basic conditions, this was an important precedent. If etoposide was epimerized at C-2 prior to reduction, the final product of the synthetic scheme would have been the cyclic ether analogue of **5**. The general inactivity of picro compounds in biological screens has been noted.[4] It was therefore especially important to establish the trans relationship of the protons at C-2 and C-3 of ring D in the ether (**3**).

Previous work by Brewer et al.[18] had shown $J_{2,3}$ of several podophyllotoxin analogues to be on the order of 13–14 Hz. Picropodophyllotoxin, in contrast, had a coupling constant of only 9 Hz. In our studies, $J_{2,3}$ of the picro analogue (**5**) of etoposide was shown to be 9.4 Hz.

Since the coupling patterns of H-2 and H-3 were complex in the ether (**3**), a parallel synthesis was carried out using lithium aluminum deuteride. This simplified the pattern for H-2 to a double doublet, since the H–D couplings were extremely small. This gave $J_{2,3}$ directly upon irradiation of H-1 (Figure 1). $J_{2,3}$ was determined in both **4** and **3** in this fashion and was found to be 13 Hz for the ether compound, thus indicating the trans relationship of H-2 and H-3.

Further chemical evidence for trans fusion of ring D was gained by reducing **5**[10] with LAH in THF, to produce the picro analogue of **4**. This product was distinct from **4** by TLC and had a mass spectrum that exhibited identical ions to those of **4** but with significantly different intensities.

Thus, by chemical and spectroscopic evidence the reduction of etoposide with LAH was shown to proceed with retention of stereochemistry at C-2. Since there was no possibility for inversion at C-3 in etoposide, the absolute stereochemistry of the products is analogous to the starting compound.

Brewer et al.[18] assigned all resonances associated with the aglycon moiety of etoposide in their 360-MHz ^1H NMR study. Protons in the glycoside moiety of etoposide were not assigned. Use of 470-MHz NMR revealed one glycoside resonance previously obscured by the large methoxy peak at δ 3.75. Double irradiation experiments have allowed us to assign all of the glycoside resonances in etoposide and the derivatives considered here. In this regard, comparison of etoposide with epipodophyllotoxin (podophyllotoxin with the opposite configuration of the hydroxy at C-4) reveals that all protons in the aglycon moiety, except for H-11, have almost identical chemical shifts. Brewer has suggested[18] that this may indicate a favoring of a conformation where the sugar rings are swung out over

Table III. Biological Test Data for Etoposide (1) and Its Derivatives in L1210 Mouse Leukemia in Vivo[a]

	% increase in life span		
dose, mg/kg	1	4	3
30	110	0	10
45	b	0	25
60	103	5	30

[a] Every data point is the average for two female mice, strain C57BL/6XDBA/2F1, inoculated with 10^5 cells, L1210. Drugs administered in Me_2SO, control mice survived 10 days. [b] Two long-term survivors were observed.

Table IV. Biological Test Data for Etoposide (1) and Its Derivatives in L1210 Mouse Leukemia in Vitro[a]

dose,	% survival			
μg/mL	control	1	4	3
0.5	100	0	357	165
5.0	100	1	221	0
50.0	100	0	43	0

[a] Cytotoxicity was studied using a continuous mouse leukemia L1210 cell line in a soft agar clonogenic assay system[22] similar to that described by Hamburger and Salmon.[23]

ring D for etoposide. In **5**, such a conformation would be discouraged, since ring D is flipped upward toward the glucose residue. The rather large shift of the anomeric proton (H-g1) by 0.9 ppm upfield in **5** may support Brewer's suggestion. The anomeric proton in **5** would spend more time in conformations over the aromatic rings and could experience shielding by the benzene ring electrons. Not only is H-g1 appreciably shifted upfield, but also H-g3 (by 0.14 ppm) and H-g5 (by 0.16 ppm) are shifted. These shifts further support the assignments made based on double irradiation experiments. That is, H-g3 and H-g5 are the only other protons on the same face of the sugar "trans-decalin" type ring system as H-g1, existing in a 1–3 diaxial relationship.

Biological Results. Etoposide is one of the most active agents tested in vivo in the mouse leukemia L1210 system.[3] Therefore, this system was chosen to test the diol (**4**) and ether (**3**) analogues vs. etoposide. The results are reported in Table III. As expected, etoposide was very active in the assay. The diol was essentially inactive in this system. The ether retained activity, but the activity was much reduced compared to the parent drug. These results were reflected in an in vitro cytotoxicity assay of L1210 cells (Table IV). Etoposide was very active in this assay, the diol was essentially inactive except at the highest dose (50 μg/mL) used, and the ether retained some activity relative to the parent drug.

These results suggest that the lactone moiety of etoposide is not an absolute requirement for cytotoxic activity. However, alteration of the lactone considerably reduces the activity of the parent drug. This result is similar to that obtained by Gensler et al.[9] with the cyclic ether analogue of podophyllotoxin where the ether analogue was not as active as the parent compound.

Experimental Section

Materials. Etoposide was generously provided by Drs. H. Friedli and H. Stahelin of Sandoz, Basle, Switzerland, and by Dr. R. L. Buchanan of Bristol Laboratories, Syracuse, NY. The purity of this material was checked by TLC and HPLC as described previously.[10,21] The picro analogue (**5**) of etoposide was prepared as described previously.[10] Solvents used were generally distilled in glass grade from Burdick and Jackson (Muskegon, MI). Pyridine (Gold Label), 95%+, lithium aluminum hydride, 98% *p*-toluenesulfonyl chloride, and calcium hydride were obtained from Aldrich Chemical Co. (Milwaukee, WI). Other inorganic reagents of analytical grade were obtained from standard suppliers. Lithium aluminum deuteride (99 atom %) was obtained from Merck Isotopes (St. Louis, MO). Silica gel preparative TLC plates were purchased from Analtech (Newark, DE). Analytical TLC plates with fluorescent indicators were acquired from J. T. Baker Chemical Co. (Phillipsburg, NJ), and analytical reverse-phase TLC plates (KC18) were purchased from Whatman (Clifton Park, NJ).

Proton survey spectra at 470 MHz were obtained on a Nicollet NTC 470 FT NMR spectrometer, interfaced to a Nicollet 1185 computer. The spectral width was 5000 Hz with 32K data points. Homonuclear decoupling experiments were carried out at low powers optimized to irradiate only the peaks of interest. Typically, a 4-mg sample was dissolved in 0.5 mL of $CDCl_3$ and filtered prior to analysis.

IR spectra (KBr) were recorded on a Beckman IR33 or IR4230, and UV spectra were obtained in methanol on a Varian-Cary 219 spectrophotometer.

Accurate-mass electron-impact mass spectra were automatically recorded on a Kratos MS50-DS55 high-resolution double-focusing mass spectrometer–computer system, operating at a dynamic resolution of 10000. An ion-accelerating potential of 8 kV, ionizing electrons of 70 eV, and a source and probe temperature of 250 and 300 °C, respectively, were used.

Synthesis of 4. All glassware was flame dried just prior to use. Tetrahydrofuran (THF) was distilled after refluxing over calcium hydride for 2 h or was used from a fresh bottle. Lithium aluminum hydride (LAH; 96 mg, 2.53 mmol) was added to THF (7.5 mL) in a 50-mL round-bottom flask. Etoposide (300 mg, 0.508 mmol) in THF (12 mL) in a test tube was added slowly over 5 min by pipet to the round-bottom flask with stirring, at room temperature. The flask was fitted with a reflux condenser and drying tube and was brought to reflux for 30 min. It was then cooled in an ice bath, and 7.5 mL of ethyl acetate (EtOAc) was dripped in slowly with swirling. The suspension was pipetted rapidly into 30 mL of a pH 1.5 buffer of 1 M HCl, 1 M sodium acetate, which raised the buffer pH to 4.5. The resulting suspension was extracted with 300 mL of EtOAc for 15 min, the extract was allowed to settle, and the aqueous phase was separated and discarded. The organic layer was washed with 100 mL of 1 M pH 7.2 phosphate buffer, dried over sodium sulfate, and reduced in volume in vacuo at 40 °C to 10 mL. Finally, *n*-hexane was gently layered onto the EtOAc in small portions with swirling after each addition, until the product was fully precipitated as a white powder. The product was dried by filtration on a Hirsch funnel. Two separate runs yielded 194 and 195 mg (0.33 mmol) of **4**, respectively (65%). The purity was greater than 90% as determined by HPLC.[10]

Purification of 4. The product (194 mg, 0.33 mmol) was dissolved in methanol (1.5 mL) and the solution was streaked onto two 20 × 20 cm 1500-μm silica gel prep TLC plates, which had been prerun in the developing solvent and oven dried after air drying. The streaked area was dried by gentle heating with a blow dryer. Since cracking of the streaked area was observed, developing solvent was streaked over the area, thoroughly saturating it, but maintaining a narrow band, and the plate was quickly transferred to a developing tank. Good resolution was obtained. The lowest band (**4**) was removed by scraping, with care being taken to avoid the faint band running just in front of it. The solvent used was EtOAc/MeOH (20:3). Analytical TLC (silica gel) showed three spots (only by very heavy spotting) at R_f 0.23 (**4**), 0.30 (faint), 0.55 (starting material, also faint). Spraying with ceric reagent visualized **4** as a light violet color.[10]

The product was recovered by transferring the silica gel to a glass-stoppered 250-mL Erlenmeyer flask. The silica was wetted with distilled water to a pasty consistency, and 190 mL of chloroform was added. Ten milliliters of MeOH was layered on top of this, and the flask was stoppered and vigorously shaken for 15 min. The suspension was vacuum filtered through Whatman

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no. 40 paper, and the filtrate was reduced in volume to a few milliliters in vacuo at 40 °C. This was rediluted with 200 mL of $CHCl_3$ and reduced in volume to about 5 mL. This volume was then filtered through a 0.5-μm MF millipore filter in a syringe filtration apparatus to eliminate any remaining silica gel. The product, 124 mg (0.21 mmol) of pure **4**, was precipitated as before with *n*-hexane (chromatographic yield 69%; overall synthetic yield 45%).

The product was pure by TLC and HPLC.[10,21] The EI mass spectrum of **4** showed *m/z* 386 (20%, the aglycon fragment, $C_{21}H_{22}O_7$) 356 (85%, $M^{+\cdot}$ – glucoside OH – CH_2 = O), and 325 (*m/z* 356 – $CH_2OH\cdot$, stable even electron ion and base peak). The accurate masses of these ions were determined by mass spectrometry and were all accurate to within 10 parts per million of the suggested compositions. (There was not enough material available after biological testing for satisfactory elemental analysis of **4** or **3**.) The 470-MHz ^1H NMR data ($CDCl_3$) are reported in Tables I and II. The ^1H NMR was consistent with the proposed product. IR (KBr) verified the absence of the carbonyl group at 1775 cm^{-1}.

Synthesis of 3. All glassware was flame dried prior to use. Pyridine was distilled over calcium hydride prior to use. Tosyl chloride (TsCl) was purified before use. A solution of 26 mg/mL of the fresh TsCl in pyridine was made up. Pure, silica-free **4** (134 mg, 0.226 mmol) was added to pyridine (1.67 mL) in a 15-mL conical test tube with a small stirring bar. An equal volume of the TsCl/pyridine solution was added, and the reaction was stirred at 5 °C in a refrigerator. TsCl (43 mg, 0.226 mmol) was added at 48 h and again at 70 h. The reaction was stopped at 80 h by adding the mixture directly to 34 mL of 2 N HCl, which precipitated the products. Extraction with 170 mL of EtOAc was carried out immediately for 5 min, and the extract was washed with an equal volume of water. The EtOAc was then dried over sodium sulfate and reduced in volume in vacuo at 40 °C. The product was precipitated with *n*-hexane as before, yielding 85 mg (0.148 mmol) of crude product. Analytical TLC (EtOAc/MeOH, 20:3) showed four spots at R_f 0.23 (starting material), 0.46 (**3**), 0.51 (competing aglycon product, which is only apparent after addition of the 3rd equiv of TsCl), and 0.61 (minor). HPLC analysis (methanol/water, 55:45 or 70:30, μBondapak C_{18} column, 1.0 mL/min) showed about 50% of **3**, assuming a similar absorbance at 288 nm for the compounds.

The mixture was streaked in MeOH onto two 1000-μm TLC plates as before. The separation of the closely traveling product at R_f 0.51 (analytical TLC) was difficult to achieve. Enrichment was obtained, to about 80%, but smaller batches of the material (~25 mg, 0.043 mmol) were rechromatographed on 250-μm silica gel Redi-plates to achieve purification to 97%. These plates also seemed more activated. In each of the prep TLC steps isolation was by swirling in MeOH. Final purification to free the product from any silica gel was again done by filtration through a 0.5-μm MF-millipore filter. About 20 mg (0.035 mmol) of 97% purity material was collected for analysis and biological testing (overall synthetic yield 15%). The ether (**3**) and the competing product at R_f 0.51 were later well separated by analytical TLC on K-C18 reverse-phase plates, appearing at an R_f of 0.50 and 0.19, respectively. Thus, preparative reverse-phase TLC or HPLC would now be a preferred procedure.

The mass spectrum of **3** contained ions at *m/z* 574 ($M^{+\cdot}$, 55%) and 368 (the aglycon fragment and base peak, $C_{21}H_{16}O_6$). High-resolution mass data: *m/z* 574 (calcd, 574.2050; found, 574.2019); 470-MHz ^1H NMR data are reported in Tables I and II. The ^1H NMR confirmed the dehydration to form the trans cyclic ether. IR (KBr) again confirmed the absence of the carbonyl group.

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β-Carbolines: Synthesis and Neurochemical and Pharmacological Actions on Brain Benzodiazepine Receptors

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We have prepared a series of tetrahydro-β-carbolines (THβC), β-carbolines (β-C), and other nitrogen heterocycles and evaluated them in vitro with respect to their ability to bind to benzodiazepine receptors. The fully aromatic β-C's were more potent than their corresponding THβC derivatives. When substituents possessing a carbonyl (CO_2Me, $COCH_3$, CHO) were introduced at the β-C 3-position the in vitro potency was augmented. Alcohol substituents (CH_2OH, $CHOHCH_3$) demonstrated decreased in vitro potency. The importance of the carbonyl moiety was further demonstrated when β-carboline-3-carboxylic acid was shown to bind tighter to benzodiazepine receptors at lower pH. A lower pH increases the concentration of the acid and decreases the concentration of the anion. 3-(Hydroxymethyl)-β-carboline (**24**), 3-formyl-β-carboline (**25**) and 3-acetyl-β-carboline (**27**) were benzodiazepine antagonists in vivo. Methyl isoquinoline-3-carboxylate (**31a**) also had in vitro activity. The same structure–activity relationships seen in β-C's were also observed for isoquinolines.

The discovery of high affinity, saturable, and stereospecific receptors for benzodiazepines in the mammalian central nervous system has led to an intensive search for endogenous factors that physiologically regulate this receptor.[1,2] Although Nielsen and co-workers[3,4] originally

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