4-aminobenzanilides showing the highest potency and the 2-aminobenzanilides the least. The 2,6-dimethylaniline derivative is the most potent anti-MES agent in both the 4- and 3-aminobenzanilides. Furthermore, although no quantitative data were measured in the 2-amino series of compounds, the 2,6-dimethylaniline derivative was one of the more active compounds in this group.

Experimental Section

Melting points were determined in open glass capillaries using a Thomas-Hoover melting point apparatus and are uncorrected. IR spectra were recorded in chloroform solutions in matched sodium chloride cells or as fluorocarbon mulls by use of a Beckman 4230 spectrophotometer. All ^1H NMR spectra were measured in $CDCl_3$ on a Varian T-60A spectrometer with an internal standard of tetramethylsilane. Elemental analyses (C, H, N) were performed by Atlantic Microlab Inc., Atlanta, GA, and the results obtained were within ±0.4 of the calculated percentage. The following experimental procedures are representative of the general procedures used to synthesize all of the compounds. Experimental data for the 2-amino- and 3-aminobenzanilides are provided in Tables I and II.

Nitrobenzanilides. A solution of the appropriate alkylaniline (0.03–0.07 mol) in 35 mL of tetrahydrofuran was added to 200 mL of 20% (w/v) aqueous potassium carbonate contained in a 1-L three-necked flask equipped with a magnetic stirrer, reflux condenser, addition funnel, and a heating mantle. A solution of nitrobenzoyl chloride (2-fold molar excess) in 35 mL of tetrahydrofuran was added dropwise, and the resulting mixture was refluxed for 12 h and maintained at or above pH 8 during the reaction period. The solution was then cooled to room temperature and extracted with chloroform (3 × 100 mL). The extracts were combined, dried over magnesium sulfate, and evaporated. The resulting residues were purified by recrystallization from benzene.

Aminobenzanilides. A solution of 5.0 g of the appropriate nitrobenzanilide in tetrahydrofuran or absolute ethanol was added to a Paar hydrogenation bottle along with 250 mg of 5% palladium on carbon. The mixture was subjected to low-pressure hydrogenation (45 psi) for 3 h, and the contents of the bottle were filtered through Celite. The filtrate was evaporated and the resulting residue was purified by recrystallization from benzene or benzene–petroleum ether (30–60 °C) mixtures.

Pharmacology. Initial anticonvulsant evaluation of these compounds was conducted using at least three dose levels (30, 100, 300 mg/kg) and in some cases a fourth dose of 600 mg/kg. All tests were performed using male Carworth Farms number-one mice. Test solutions of all compounds were prepared in 30% polyethylene glycol 400, and animals were dosed intraperitoneally 30 min prior to testing.

MES seizures were elicited with a 60-cycle alternating current of 50-mA intensity delivered for 0.2 s via corneal electrodes. A drop of 0.9% saline was instilled in the eye prior to application of electrodes. Abolition of the hind limb tonic extension component of the seizure was defined as protection in the MES test.

The scMet seizure threshold test was conducted by administration of 85 mg/kg of pentylenetetrazole as a 0.5% solution in the posterior midline. Protection in this test was defined as a failure to observe a single episode of clonic spasms of at least 5-s duration during a 30-min period following administration of the test compound.

Neurological deficit was measured in mice by the use of the rotorod test. The dosed animal was placed on a 1-in.-diameter knurled plastic rod rotating at 6 rpm. Neurologic toxicity was defined as the failure of the animal to remain on the rod for 1 min. The median anticonvulsant potency (ED50) and toxicity (TD50) were determined by the graphical method.

Acknowledgment. We deeply appreciate the assistance of the Anticonvulsant Drug Development Program, Epilepsy Branch, NINCDS, in the pharmacological testing of these compounds. The help of Gill D. Gladding of the ADD Program is gratefully acknowledged.

Registry No. 1, 4424-17-3; 1a, 2385-27-5; 2, 4943-85-5; 2a, 2385-25-3; 3, 22312-62-5; 3a, 50623-64-8; 4, 32212-38-7; 4a, 50623-00-2; 5, 35703-71-0; 5a, 102630-94-4; 6, 21132-02-5; 6a, 102630-95-5; 7, 102630-80-8; 7a, 102630-96-6; 8, 13922-38-8; 8a, 13922-37-7; 9, 102630-81-9; 9a, 102630-97-7; 10, 102630-82-0; 10a, 102630-98-8; 11, 102630-83-1; 11a, 102630-99-9; 12, 102630-84-2; 12a, 102631-00-5; 13, 102630-85-3; 13a, 102631-01-6; 14, 14315-16-3; 14a, 2243-73-4; 15, 14315-20-9; 15a, 102631-02-7; 16, 14315-23-2; 16a, 69754-50-3; 17, 14315-26-5; 17a, 6911-92-8; 18, 102630-86-4; 18a, 102631-03-8; 19, 102630-87-5; 19a, 102631-04-9; 20, 102630-88-6; 20a, 102631-05-0; 21, 14635-96-2; 21a, 102631-06-1; 22, 102630-89-7; 22a, 102631-07-2; 23, 102630-90-0; 23a, 102631-08-3; 24, 102630-91-1; 24a, 102631-09-4; 25, 102630-92-2; 25a, 102631-10-7; 26, 102630-93-3; 26a, 102631-11-8; aniline, 62-53-3; 2-methylaniline, 95-53-4; 3-methylaniline, 108-44-1; 4-methylaniline, 106-49-0; 2,3-dimethylaniline, 87-59-2; 2,4-dimethylaniline, 95-68-1; 2,5-dimethylaniline, 95-78-3; 2,6-dimethylaniline, 87-62-7; 3,4-dimethylaniline, 95-64-7; 3,5-dimethylaniline, 108-69-0; 2-isopropylaniline, 643-28-7; 2-isopropyl-6-methylaniline, 5266-85-3; 2-isopropyl-6-ethylaniline, 53443-93-9; 2-nitrobenzoyl chloride, 610-14-0; 3-nitrobenzoyl chloride, 121-90-4.

3,17β-Dihydroxy-20,21-epoxy-19-norpregna-1,3,5(10)-trienes: Synthesis, Rearrangement, Cytotoxicity, and Estrogen-Receptor Binding

Julie C. Gill, Peter M. Lockey, Brian A. Marples,* and John R. Traynor*

Department of Chemistry, University of Technology, Loughborough, Leicestershire, LE11 3TU, U.K.
Received November 19, 1985

Diastereoisomers of 3,17β-dihydroxy-20,21-epoxy-19-norpregna-1,3,5(10)-triene have been prepared as potential antitumor agents. Both isomers undergo the base-catalyzed Payne rearrangement. The isomers were cytotoxic to mammalian cells in culture and were able to displace [^3H]estradiol from binding sites in rat uterine cytosols with $^1/_7$ and $^1/_{70}$ the potency of estradiol. The reasons for this difference are discussed.

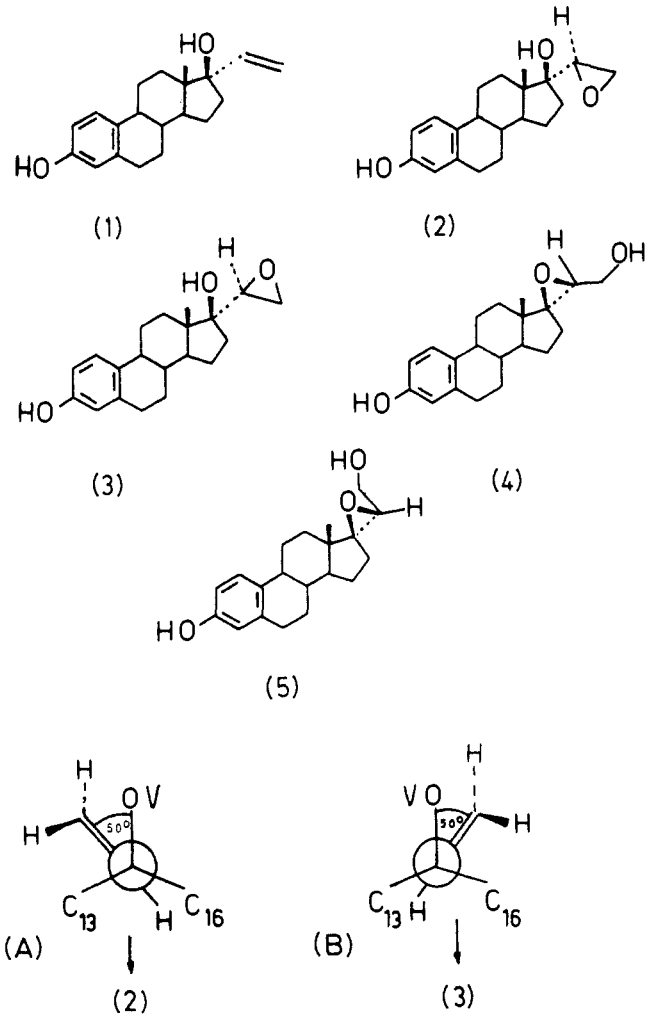
Estrogen analogues bearing an alkylating function have been synthesized by several groups in attempts to obtain "receptor-carried cytotoxic agents" active against estrogen-dependent tumors.[1-3] Unfortunately, introduction of the alkylating function into an estrogen often leads to a loss of estrogen-receptor affinity.[4]

It is known from structure–activity studies that the estrogen receptor is relatively tolerant to substitutions at the estrogen 17α-position. Thus ethynylestradiol has a

(1) Chagonda, L. S.; Lockey, P. M.; Marples, B. A.; Traynor, J. R. *Steroids* **1984**, *43*, 283.
(2) Katzenellenbogen, J. A.; McGorrin, R. J.; Tatec, T.; Kempton, R. J.; Carlson, K. E.; Kinder, D. H. *J. Med. Chem.* **1981**, *24*, 435–450.
(3) Leclercq, G.; Henson, J.-C. *Cancer Treat. Rep.* **1978**, *62*, 1255–1257.
(4) *Cytotoxic Estrogens in Hormone Receptive Tumors*; Raus, J., Martens, H., Leclercq, G., Eds.; Academic: London, 1980.

0022-2623/86/1829-1537$01.50/0

Scheme I



greater affinity for the receptor than estradiol itself, and introduction of a 17α-methyl group reduces estradiol binding by only 13%.[5] In this paper we report the synthesis and in vitro evaluation of two 3,17β-dihydroxy-20,21-epoxy-19-norpregna-1,3,5(10)-triene isomers (**2** and **3**) as potential target-selective agents against breast tumors. We have chosen the epoxide group as the alkylator for several reasons. It is relatively small and may disturb receptor binding less than, for example, a mustard group. It has relatively low activity, which should reduce the potential for unselective alkylation during passage to the target cell. Furthermore, oxygenation of the 17α-ethynyl group has been postulated as an important metabolic pathway in primates, including humans, which could be of particular interest with regard to a role in cytotoxicity and carcinogenicity.[6] It is not unreasonable that electrophilic species such as **2** and **3** may be implicated, though there is no direct evidence for this.

Chemistry. Oxidation of 17α-vinylestradiol (3,17β-hydroxy-19-norpregna-1,3,5(10),20-tetraene,[7] **1**) with *t*-BuOOH/VO(acac)$_2$ gave a 65:35 mixture[8] of the (20*R*)- and (20*S*)-20,21-epoxides **2** and **3**, which were separated by fractional crystallization. The assignment of the 20*R* configuration to the major product follows from arguments

Scheme II

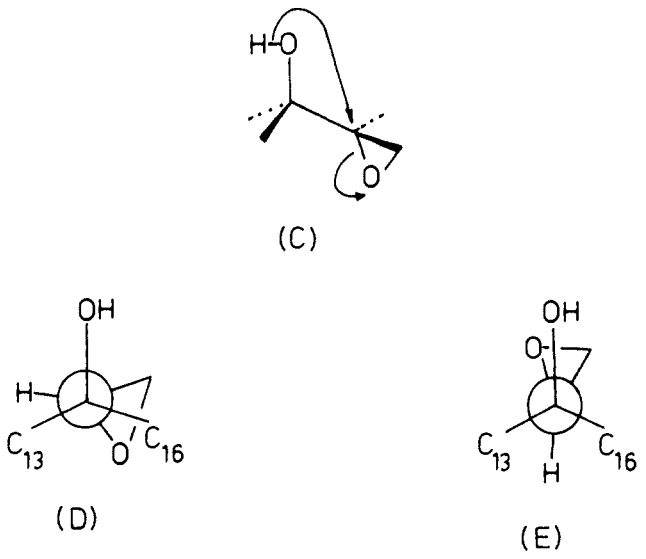


Table I. Effects of 20,21-Epoxy-19-norpregnatrienes on Cell Growth

compound	inhibition of cell growth: IC_{50},[a] M × 10^{-6} HeLa S3	GH3
(20*R*)-20,21-epoxide **2**	6.5 ± 1.9 (**4**)	5.5 (2)
(20*S*)-20,21-epoxide **3**	18.6 ± 2.0 (**4**)	17.7 (2)
elephantopin	0.64 ± 0.01 (3)	nt
vernolepin	3.5 ± 0.1 (3)	5.2 (2)
estramustine	3.4 ± 0.2 (3)	nt[b]

[a] Results represent means ± SEM. Values in parentheses are number of determinations. [b] Not tested = nt.

similar to those presented by Sharpless and co-workers.[9] Accordingly, it is assumed that the transition state A is preferred to B because, in the latter, there is a significant steric interaction between the 20-H and the 12α-H (Scheme I).

The configurational assignments are further supported by the differential ease of Payne rearrangement[10] of the epoxides. The (20*R*)-20,21-epoxide **2** readily rearranges in MeOH/K_2CO_3 to the (20*S*)-17β,20-epoxide **4** while the (20*S*)-20,21-epoxide **3** does not rearrange under these conditions. However, in the presence of *t*-BuOK/*t*-BuOH, the rearrangement proceeds to give the (20*R*)-17β,20-epoxide **5**. It would be anticipated that the conformation C, in which the OH group is antiperiplanar to the C(20)–O bond of the epoxide, would be favored for the Payne rearrangement. Such a conformation would be most easily attained by the (20*R*)-20,21-epoxide **2** (D, Scheme II) since the 21-methylene group is relatively unhindered by the 16-methylene group. In the (20*S*)-20,21-epoxide **3** the antiperiplanar conformation would involve considerable steric hindrance between the 21-methylene group and the 12α-hydrogen, and conformation E (Scheme II) is likely to be preferred (see later discussion).

Biological Results and Discussion

The 20,21-epoxides **2** and **3** were evaluated for cytotoxicity against HeLa S3 cells (human cervical carcinoma) and GH3 cells (rat pituitary adenoma). GH3 cells, unlike HeLa S3, contain estrogen receptors and will concentrate

(5) Korenman, S. G. *Steroids* **1969**, *13*, 163.
(6) Hilton, E. D.; Williams, M. G.; Goldzieher, J. W. *Steroids* **1977**, *30*, 71.
(7) A. G. Schering, Ger. Patent 870 099; *Chem. Abstr.* **1952**, *52*, P15606a.
(8) Determination by integration of the triplets ($J \sim 3.5$ Hz) at δ 3.22 (**2**) and 3.34 (**3**) for the 20-H in the ^{1}H NMR spectrum.
(9) (a) Rossiter, B. E.; Verhoven, T. R.; Sharpless, K. B. *Tetrahedron Lett.* **1979**, 4733. (b) Miyano, S.; Lu, L. D.-L.; Viti, S. M.; Sharpless, K. B. *J. Org. Chem.* **1983**, *48*, 3607.
(10) (a) Payne, G. B. *J. Org. Chem.* **1962**, *27*, 3819. (b) Behrens, C. H.; Sharpless, K. B. *Aldrichimica Acta* **1983**, *16*, 67, and references cited therein.

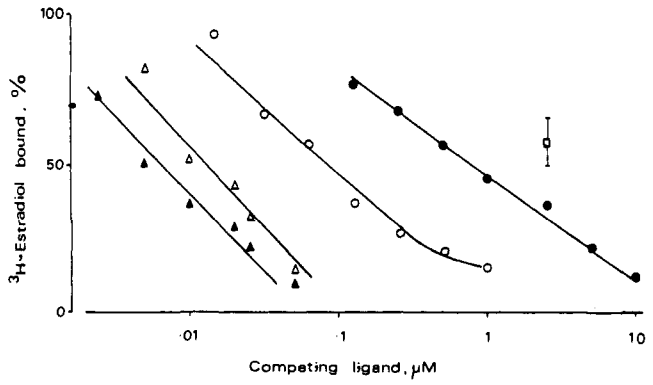


Figure 1. Displacement of specifically bound [^{3}H]estradiol from rat uterine cytosols by 20,21-epoxy-19-norpregnatrienes **2** (○) and **3** (●), estradiol (△), diethylstilbestrol (▲), and estramustine (□). Points represent the means of three experiments.

[^{3}H]estradiol from medium supplemented with ^{3}H-labeled hormone, although cell growth is not estrogen-dependent.[11] Both compounds were toxic to either cell line, though **2** was the more active (Table I). A study of the reactions of **2** and **3** with model nucleophiles is currently under way to investigate this differential cytotoxicity. The compounds were much less active than the highly potent sesquiterpene elephantopin but of the same degree of activity as the sesquiterpene vernolepin and the estrogen–mustard derivative estramustine. No selectivity for the estrogen-receptor-containing GH3 cell line was observed.

As a further indication of the potential for selectivity toward estrogen-dependent tumors, an estrogen-receptor competitive binding assay was employed, using cytosols prepared from immature rat uteri. Although both isomers were able to displace [^{3}H]estradiol (Figure 1), **2** (IC_{50} = 79.0 ± 11 nM (n = 3)) was 10 times more effective than **3** (IC_{50} = 724 ± 89 nM (n = 3)). By comparison, the IC_{50} for estradiol itself was 10.6 ± 0.6 nM (n = 3), for diethylstilbestrol it was 5.6 ± 0.1 nM (n = 3), and for estramustine it was approximately 2.5 μM, 30 times less active than **2**. The difference in potency between the isomers **2** and **3** can probably be ascribed to the disruption of H bonding to the estrogen receptor of the 17β-hydroxyl in **3** by the presence of the epoxide grouping (Scheme II). The epoxide oxygen atom may sterically inhibit such hydrogen bonding or may compete by hydrogen bonding itself to the 17β-hydroxy group.

Further support for this idea is obtained from the fact that **2** much more readily alkylates the estrogen receptor than **3**. Thus **2** binds to the receptors causing a 66 ± 16% (n = 3) loss of sites, similar to the 62 ± 16% (n = 3) alkylation we see with the affinity label ORG 4333 (11β-(chloromethyl)estra-1,3,5(10)-triene-3,17β-diol).[12] By contrast **3** is irreversibly bound to only 12 ± 7% (n = 3) of the sites.

The present work has resulted in diastereoisomers of 20,21-epoxy-3,17β-hydroxypregna-1,3,5(10)-triene. Both compounds have similar cytotoxic properties. However, the rather weak activity, confirmed by the fact that neither compound shows any reactivity with glutathione during incubation for 1 h at 37 °C,[1] could be an important property in a potentially selective antitumor drug. In addition, isomer **2** has good affinity for the estrogen receptor, showing that the presence of an alkylating function in the 17α-position is well tolerated, though steric considerations are important. This is confirmed by the ability of **2**, but not **3**, to bind irreversibly.

In spite of its apparent affinity for estrogen binding sites, compound **2** is no more toxic to the estrogen-receptor-containing GH3 cells than to HeLa S3 cells, and the small difference in activity between **2** and **3** is independent of the cell line used. These findings suggest that the estrogen-receptor mechanism is not important in the cytotoxic process in GH3 cells. The concentration of estrogenic molecules by the GH3 cells is dependent upon the estrogen-receptor population.[11] It is apparent that any intracellular **2** that results from this process is insufficient to cause significant cell death. The cytotoxicity of these compounds is then a nonspecific process brought about by higher drug levels when numbers of drug molecules greatly exceed the receptor population. Also, if receptor alkylation does occur, as seems the case with compound **2**, then the 17-epoxide may not be available to attack important cellular nucleophiles, in particular DNA. To test out this latter hypothesis we are currently seeking second-generation compounds, based on **2**, that have high receptor affinity but do not bind irreversibly and may then be released in the nucleus to attack DNA. However, such compounds may have to be more potent in order to achieve effective control of cell growth.

Compounds **2** and **3** are currently undergoing evaluation for their ability to react with DNA,[13] which may be more relevant to their cytotoxic properties, but which could also be an important component in the possible carcinogenicity of estrogenic molecules.

Experimental Section

The following compounds were obtained from the indicated sources: 17β-[6,7-$^3H_2$]estradiol (49 Ci/mmol, Amersham), tissue culture medium (Flow), all other reagents (Sigma or Aldrich). Elephantopin and vernolepin were kindly provided by Dr. A. T. Sneden, University of Virginia, estramustine by A. B. LeO, Helsingburg, and 11β-(chloromethyl)estra-1,3,5(10)-triene-3,17β-diol (ORG 4333) by Organon Laboratories, Surrey.

Solutions were dried over anhydrous $MgSO_4$, and solvents were removed in vacuo on a rotary evaporator. Flash chromatography was performed with Kieselgel 60 (Merck), eluting with CH_2Cl_2/EtOAc (6:1). IR spectra were determined with a Perkin-Elmer 177 spectrophotometer, UV spectra with a Pye-Unicam SP8000 spectrophotometer, and ^{1}H NMR spectra at 90 MHz with a Perkin-Elmer R32 spectrometer or at 400 MHz with a Bruker WH400 spectrometer. Mass spectra were determined with a Kratos MS80 spectrometer. Rotations were measured for dioxane solutions at ambient temperature with an AA-10 polarimeter. Melting points were determined on a Kofler hot-stage apparatus.

3,17β-Dihydroxy-20,21-epoxy-19-norpregna-1,3,5(10)-trienes (2, 3). $VO(acac)_2$ (44 mg, 0.17 mmol) was added to a stirred solution of 17α-vinylestradiol (1; 2.5 g, 8.39 mmol) in CH_2Cl_2 (500 mL) at 0 °C. Anhydrous t-BuOOH in toluene solution (4.4 mL, 3.8 M) was added dropwise, and the reaction mixture was stirred overnight while the temperature was maintained below 10 °C. The reaction mixture was washed with sodium sulfite solution and water, and the CH_2Cl_2 solution was dried and evaporated to afford a brown solid. Flash chromatography, eluting with CH_2Cl_2/EtOAc (6:1), gave a mixture (ca. 65:35) of the (20R)- and (20S)-20,21-epoxides **2** and **3** (1.8 g, 68%). Fractional crystallization of the mixture from chloroform gave the (20R)-20,21-epoxide **2** (39%): mp 154–158 °C;[14] $[\alpha]_D$ +41.8° (c 1.0); IR (KBr) 3380 (OH) cm^{-1}; ^{1}H NMR ($CDCl_3$/$(CD_3)_2CO$/D_2O) δ 0.96 (3 H,

(11) Mester, J.; Brunelle, R.; Jung, I.; Sonnenschein, C. *Exp. Cell Res.* **1973**, *81*, 447–452.
(12) Van Den Broek, A. J.; Leemhuis, J.; De Winter, M. S.; Zeelan, F. J. *Pharm. Weekbl. Sci. Ed.* **1983**, *5*, 182–183.
(13) This work is being performed by Drs. Grover and Phillips at the Chester-Beatty Research Institute, London, U.K.
(14) All of the compounds **2–5** melted over a relatively wide range, but compounds **3** and **4** melted over a particularly wide range with decomposition; the latter went through a partial resolidification before finally melting.

s, 13-Me), 2.7–3.0 (4 H, m, 6-CH_2 and 21-CH_2), 3.22 (1 H, t, J = 3.5 Hz, 20-CH), 6.6–7.3 (3 H, m, A-ring CH's); MS, 314.1880 (M, $C_{20}H_{26}O_3$). Anal. ($C_{20}H_{26}O_3$) C, H. Fractional crystallization from methanol gave the (20*S*)-20,21-epoxide **3** (11%): mp 184–200 °C dec;[14] $[\alpha]_D$ +46.6° (c 0.9); IR (KBr) 3600, 3510 and 3240, 3420 (OH) cm^{-1}; ^1H NMR ($CDCl_3/(CD_3)_2CO/D_2O$) δ 0.94 (3 H, s, 13-Me), 2.7–3.0 (4 H, m, 6-CH_2 and 21-CH_2), 3.34 (1 H, t, J = 3.5 Hz, 20-CH), 6.6–7.3 (3 H, m, A-ring CH's); MS, 314.1876 (M, $C_{20}H_{26}O_3$). Anal. ($C_{20}H_{26}O_3$) C, H.

(20*S*)-3,21-Dihydroxy-17β,20-epoxy-19-norpregna-1,3,5-(10)-triene (4). K_2CO_3 (1.31 g, 7.9 mmol) was added to a solution of the (20*R*)-20,21-epoxide **2** (1.01 g, 3.2 mmol) in methanol (69 mL). The resulting suspension was stirred overnight at room temperature during which time the suspended solid dissolved. Ethyl acetate was added to the methanol solution, and the resultant solution was washed with water, dried, and evaporated to afford a white solid (0.81 g, 80%), which on crystallization from ethyl acetate gave the (20*S*)-17β,20-epoxide **4** (0.63 g, 62%): mp 173–260 °C dec;[14] $[\alpha]_D$ +37.6° (c 0.9); IR (KBr) 3420, 3320 (OH) cm^{-1}; ^1H NMR ($(CD_3)_2CO/D_2O$) δ 0.87 (3 H, s, 13-Me), 2.6–3.0 (2 H, m, 6-CH_2), 3.17 (1 H, t, J = 5.5 Hz, 20-CH), 3.66 (2 H, m, 21-CH_2), 6.5–7.3 (3 H, m, A-ring CH's). At 400 MHz the multiplet at δ 3.66 is fully resolved into the expected 8 lines of the AB component of an ABX system (J_{AB} = 12 Hz); MS, 314.1885 (M, $C_{20}H_{26}O_3$). Anal. ($C_{20}H_{26}O_3$) C, H.

(20*R*)-3,21-Dihydroxy-17β,20-epoxy-19-norpregna-1,3,5-(10)-triene (5). KO-*t*-Bu (1.0 g, 8.20 mmol) was added to a solution of the (20*S*)-20,21-epoxide **3** (0.93 g, 2.96 mmol) in *t*-BuOH (30 mL) at 30 °C. The reaction mixture was stirred overnight while the temperature was maintained at 30–40 °C after which it was diluted with water and extracted (2×) with ethyl acetate. The combined extracts were washed with water, dried, and evaporated to afford a white solid (0.83 g, 89%), which on crystallization from ethyl acetate (2×) gave the (20*R*)-17β,20-epoxide **5** (0.37 g, 40%): mp 185–189 °C[14]; $[\alpha]_D$ +29.9° (c 1.0); IR (KBr) 3160, 3400 (OH) cm^{-1}; ^1H NMR ($(CD_3)_2CO/D_2O$) δ 0.99 (3 H, s, 13-Me), 2.6–2.9 (2 H, m, 6-CH_2), 3.09 (1 H, q, J = 4 and 7 Hz, 20-CH), 3.90 (2 H, m (8 lines), J_{AB} = 12 Hz, 21-CH_2), 6.5–7.2 (3 H, m, A-ring CH's); MS 314.1876 (M, $C_{20}H_{26}O_3$). Anal. ($C_{20}H_{26}O_3$) C, H.

Cell Culture Assay. HeLa S3 cells were cultivated in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 1% nonessential amino acids. GH3 cells were cultivated in Ham's F-10 medium supplemented with 15% donor horse serum and 2 mM L-glutamine. Both cell lines were grown in the presence of penicillin (50 IU mL^{-1}) and streptomycin (50 μg mL^{-1}). Compounds were assayed following the Cancer Chemotherapy National Service Center (CCNSC) protocol for KB cells.[15] Cell numbers were determined with use of a Coulter counter, and growth-inhibition curves were plotted from mean cell counts at 48 h. In all cases drugs were added in Me_2SO, which did not exceed a final concentration of 0.5%.

Inhibition of [^{3}H]Estradiol Binding. Immature rat uteri from estradiol benzoate treated rats (0.16 μg daily ×3) were homogenized in TED buffer (0.01 M Tris, 0.0015 M EDTA, 0.0005 M dithiothreitol, pH 7.4 at 25 °C). The homogenate was centrifuged at 100000*g* for 1 h (4 °C), and the cytosols were used immediately. Cytosol (150 μL) was incubated with different concentrations of competing ligands added in TED buffer (50 μL) and [^{3}H]estradiol (3.5×10^{-8} M, 50 μL) in TED buffer at 30 °C for 30 min. Parallel incubation of cytosol (150 μL), [^{3}H]estradiol (50 μL), and 50 μL TED or 50 μL of a solution of diethylstilbestrol in TED was used to determine the specific binding of [^{3}H]estradiol. All tubes were cooled in ice/water for 15 min, and then 200 μL of a suspension of dextran-coated charcoal (250 mg % Norit-A, 2.5 mg % dextran) in TED buffer was added and allowed to stand for 20 min in ice/water with occasional shaking. Tubes were centrifuged at 2000*g* for 5 min, and 200-μL samples of the supernatant were added to 5 mL Unisolve E (Koch-Light) and counted in a Phillips PW4700 liquid scintillation counter. Counting efficiency was 30–35%.

Reversibility of Drug–Estrogen-Receptor Binding. Cytosol aliquots (300 μL) were incubated with the test compound, at a level that displaced all specificity bound [^{3}H]estradiol, in TED buffer (100 μL) and [^{3}H]estradiol (35 nM, 100 μL) in TED buffer at 4 °C. After 16 h, dextran-coated charcoal suspension (400 μL) was added. Twenty minutes later, the tubes were centrifuged (2000*g*, 5 min), and an aliquot of the supernatant was counted for radioactivity to give a base-line level for binding. To the remaining supernatant, 20 μL of [^{3}H]estradiol (35 nM) was added, and the mixture was incubated for 20 h at 25 °C, then treated with dextran–charcoal, centrifuged as above, and the radioactivity in the supernatant counted. An increase in bound [^{3}H]estradiol after the second incubation was taken as an indication of the reversibility of binding of competing ligands. A parallel incubation containing 30 nM unlabeled estradiol to protect binding sites was included as a control on receptor denaturation during the prolonged incubations.

Acknowledgment. We thank the Cancer Research Campaign for a research studentship (to JCG) and the SERC for financial assistance in obtaining high-field ^1H NMR spectra.

Registry No. 1, 7678-95-7; **2**, 102651-47-8; **3**, 102651-48-9; **4**, 102651-49-0; **5**, 102651-50-3.

(15) *Cancer Chemother. Rep.* **1962**, *25*, 22.

Synthesis and Biological Activity of 3-Amino-5-(3,5-diamino-6-chloropyrazin-2-yl)-1,2,4-oxadiazole: An Amiloride Prodrug

Mark G. Bock,* Robert L. Smith, Edward H. Blaine, and Edward J. Cragoe, Jr.

Merck Sharp & Dohme Research Laboratories, West Point, Pennsylvania 19486. *Received December 13, 1985*

The pyrazinyl-1,2,4-oxadiazoles **4a** and **4b** were synthesized by two different approaches. The corresponding *N*-methyloxadiazolium salts **13a** and **13b** were also prepared. These compounds were evaluated for their diuretic and saluretic activity in rats and dogs. All compounds exhibited electrolyte excretion profiles similar to amiloride **1**. The facile conversion of **4a** to **1** was demonstrated chemically and in vivo in both rats and dogs.

The unique electrolyte excretion profile elicited by amiloride (**1**)[1] in experimental animals[2] has thrust it to the forefront in medicinal research.[3] Accordingly, this clinically effective,[4] potassium-sparing diuretic and its closely

0022-2623/86/1829-1540$01.50/0