83605-05-4; 48·HFu, 83605-07-6; 49·HFu, 83605-09-8; 50·HMl, 83605-11-2; 51·0.5-napthalenedisulfonic acid, 83615-42-3; (+)-52, 83605-12-3; (−)-52, 83605-14-5; (+)-52·HMl, 83605-13-4; (−)-53·HMl, 83605-15-6; phenyl propionate, 637-27-4; methyl propionate, 554-12-1; *N*-ethyl-2-methoxyaniline, 15258-43-2; propionyl chloride, 79-03-8; 3-methoxyphenethyl methanesulfonate, 40759-46-4; 2-methoxyphenethyl methanesulfonate, 83605-16-7; 2-methoxyphenethyl alcohol, 7417-18-7; ethyl acrylate, 140-88-5; 2-chlorophenethylamine, 13078-80-3; cyclohexylamine, 108-91-8; allyl bromide, 106-95-6.

Analogues of Aminoglutethimide: Selective Inhibition of Cholesterol Side-Chain Cleavage

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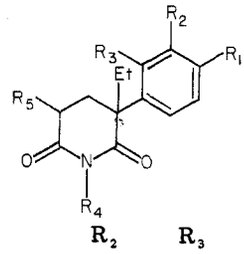
In our probing of the structural features responsible for the inhibitory activity of aminoglutethimide [1, 3-(4-aminophenyl)-3-ethylpiperidine-2,6-dione] toward the cholesterol side-chain cleavage enzyme system desmolase and the estrogen-forming system aromatase, targets in the action of 1 against hormone-dependent mammary tumors, analogues in several categories have been synthesized and evaluated. Of the known monoamino derivatives, the meta derivative [2, 3-(3-aminophenyl)-3-ethylpiperidine-2,6-dione] was as inhibitory toward desmolase as 1, and the *N*-amino analogue [4, 1-amino-3-ethyl-3-phenylpiperidine-2,6-dione] was three times as inhibitory (respective K_i values of 1, 2, and 4 are 14, 13, and 4.6 μM), but 2 was a weak inhibitor and 4 was a noninhibitor of aromatase. Another amino analogue [5, 5-amino-3-ethyl-3-phenylpiperidine-2,6-dione] inhibited neither enzyme system. Reaction of glutethimide (11) with hydrazine and thermal cyclization of the resulting amide hydrazide (15) afforded an improved synthesis of 4. Analogues having a second amino substituent, either at C-5 (10) or at N-1 (14) of the piperidine-2,6-dione residue, were less inhibitory than was 1 toward desmolase and aromatase. Among analogues having little or no inhibitory activity were hydroxy derivatives of 1 and 2, namely, 3-(4-amino-3-hydroxyphenyl)-3-ethylpiperidine-2,6-dione (20) and the 3-amino-4-hydroxy analogue (21).

Aminoglutethimide [1, 3-(4-aminophenyl)-3-ethylpiperidine-2,6-dione] was patented in 1958[2] as an anticonvulsant drug but was withdrawn in 1966, mainly because it caused adrenal insufficiency.[3] Because it inhibited adrenal steroidogenesis, 1 has found use as an alternative



	R_1	R_2	R_3	R_4	R_5
1	NH_2	H	H	H	H
2	H	NH_2	H	H	H
3	H	H	NH_2	H	H
4	H	H	H	NH_2	H
5	H	H	H	H	NH_2
6	NO_2	H	H	H	H
11	H	H	H	H	H
12	H	NO_2	H	H	H
13	H	H	NO_2	H	H
16	$NHCOCH_3$	H	H	H	H
17	NHCHO	H	H	H	H
18	OH	H	H	H	H
19	OH	NO_2	H	H	H
20	NH_2	OH	H	H	H
21	OH	NH_2	H	H	H

to adrenalectomy in the treatment of metastatic breast carcinoma.[4,5] The drug inhibits several steps in the pathways of steroidogenesis, of which the principal ones appear to be conversion of cholesterol into pregnenolone[3] (mediated by desmolase) and of androstenedione and testosterone into estrone and estradiol[6] (mediated by aromatase). It is not clear which of these two major inhibitory activities of 1 is most important in determining clinical response. Blockade of the desmolase step in humans appears to be incomplete, since levels of Δ^4-steroids (progesterone, 17α-hydroxyprogesterone, and androstenedione) are actually enhanced during the first 2 weeks of therapy and are only latterly depressed below basal levels.[7] The fall in estrogens (estrone and estradiol) is immediate, and it may be that this effect on the aromatase system is the clinically relevant action, since estrogens may well be of greater relevance to tumor growth in vivo than other steroids. However, it has been suggested[7] that the initial increase in Δ^4-steroids results from a stimulation of the action of the 3β-ol dehydrogenase Δ^5–Δ^4-isomerase complex by the drug, resulting in preferential conversion of Δ^5-steroid precursors into progesterone, and further,[8] that the resulting combined effects of estrogen suppression and androgen preservation both contribute to tumor regression, since, in postmenopausal women, androgen administration may ameliorate growth of breast carcinomas.[9] Comparison of 1 and its analogues with 4-hydroxyandrostenedione[10] and its congeners having activity against aromatase but

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not against desmolase could help to clarify the relative importance of the two modes of inhibition in the antitumor activity of **1**, as would the development of an analogue having inhibitory activity toward desmolase, but not aromatase. In this connection, the investigations[11] into the inhibition of cholesterol side-chain cleavage by the steroidal inhibitor 22-azacholesterol and its analogues is complementary to our approach of attempting to segregate this inhibitory action in analogues of the nitrogenous inhibitor **1**.

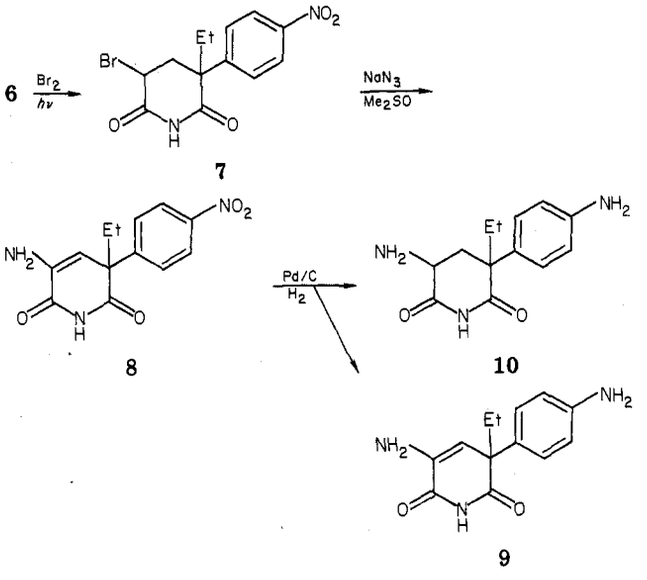
Few guidelines are available from which the structural features favorable for desmolase or aromatase inhibition can be deduced. Analogues of **1** have been tested for their ability to induce abortions in pregnant rats.[12] Pregnancy is maintained by plasma progesterone, and **1** itself apparently induces abortions by inhibiting desmolase and, hence, depleting progesterone precursors. However, the analogues were inactive, and the structural variations represented (modification of the *p*-amino function by acylation or Schiff base formation, its replacement by NO_2 or H, or N-alkylation of the glutarimide ring nitrogen) are presumably unfavorable for inhibiting desmolase activity. Aminoglutethimide shows type II binding to cytochrome P_{450} in mitochondria from bovine corpus luteum, which is characteristic of nitrogenous inhibitors of cytochrome P_{450} type enzymes.[13] Hence, it is likely that the amino or other basic function is necessary for inhibitory activity toward desmolase.

The present study emphasizes the effect on desmolase and aromatase inhibitory activity of relocating the amino group, and in this context the known monoaminoglutethimides (**1**, **2**, **4**, and **5**) have been investigated. We have also investigated analogues having a second amino substituent, known metabolites of **1** and compounds having a hydroxy substituent in place of, or in addition to, the amino function. These last derivatives were of interest because they have the hydrogen bonding, though not the basic properties, associated with the amino group and because hydroxy derivatives are potential (though not proven) metabolites of the aminoglutethimides.

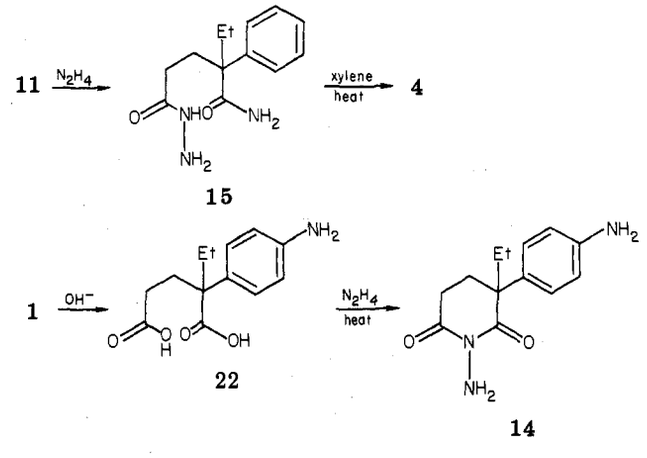
Results and Discussion

Synthesis of Analogues. The preparation of pure *p*-nitroglutethimide [**6**, 3-ethyl-3-(4-nitrophenyl)-piperidine-2,6-dione], the starting material for the synthesis (Scheme I) of 5-amino-3-(4-aminophenyl)-3-ethylpiperidine-2,6-dione (**10**), proved unexpectedly difficult. Mixtures of isomers were generally isolated when published procedures[2,14] were followed. It was recently shown[15] that the major byproduct in the nitration of glutethimide (**11**, 3-ethyl-3-phenylpiperidine-2,6-dione) had been wrongly ascribed the structure **13** and was, in fact, the meta analogue (**12**), the ortho derivative (**13**) constituting only ~1% of the mononitro derivatives formed under standard conditions. We have developed a convenient one-step procedure, namely, N-methylation, to convert the mixture of nitro compounds into derivatives separable by GLC and used it to analyze the reaction products and to monitor the purification of individual isomers by fractional crystallization. The yield of the ortho isomer (**13**) could be improved, but attempts to prepare the hitherto undescribed o-amino derivative (**3**) by reduction of **13** were unsuccessful.

Scheme I



Scheme II



Synthesis[16] of **4** and of 1-amino-3-(4-aminophenyl)-3-ethylpiperidine-2,6-dione (**14**) (Scheme II) by pyrolysis of the hydrazinium salt of the appropriate diacid afforded poor yields. In an improved synthesis of **4** (Scheme II), scission of the glutarimide (piperidine-2,6-dione) ring by hydrazine was followed by thermal ring closure of the resulting amide hydrazide (**15**).

Enzyme-Inhibitory Activity of Aminoglutethimide and Analogues. Table I expresses the inhibitory potency toward desmolase and aromatase for a group of analogues in which the amino substituent in the para position has been modified or replaced by a different group or in which there is a substituent additional to the *p*-amino group. Since all these analogues were poor inhibitors, the data presented refer only to the maximum concentration of the analogue examined in the assays. For comparison, the inhibition exerted by **1** under the same experimental conditions has been included.

The lack of desmolase inhibition by **11** and *N*-acetylaminoglutethimide [**16**, 3-[4-(acetylamino)phenyl]-3-ethylpiperidine-2,6-dione] is consistent with previous observations on their effects on steroidogenesis. Thus, **11**

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Table I. Extent of Inhibition of Desmolase and Aromatase by Weakly Inhibitory or Noninhibitory Analogues of Aminoglutethimide at Maximum Concentrations Tested

no.	compd	% inhibn of desmolase (inhibitor concn 50 μg/mL)	% inhibn of aromatase (inhibitor concn 20 μ/mL)
1	*p*-aminoglutethimide	85	90
11	glutethimide	none	none
18	*p*-hydroxyglutethimide	none	none
6	*p*-nitroglutethimide	20	20
16	*N*-acetylamino-glutethimide	none	none
17	*N*-formylamino-glutethimide	12	18
19	*p*-hydroxy-*m*-nitroglutethimide	none	none
20	*p*-amino-*m*-hydroxyglutethimide	none	none
21	*m*-amino-*p*-hydroxyglutethimide	35	none

Table II. Apparent K_i Values for Strong Inhibitors of Desmolase[a]

no.	compd	apparent K_i, μM
1[b]	*p*-aminoglutethimide	14
2	*m*-aminoglutethimide	13
4	*N*-aminoglutethimide	4.6

[a] Competitive inhibition: K_m = 4.5 μM [26-^{14}C]-cholesterol. [b] K_i for aromatase (K_m = 0.13 μM [1β,2β-^{3}H]testosterone) = 0.6 μM.

was 100 times less active than 1 in inhibiting the conversion of cholesterol to pregnenolone in acetone powders of human and rat adrenal homogenates.[17] In bovine adrenal cell cultures, whereas 10 μg/mL of 1 reduced glucocorticoid output to 8.3% of resting levels, **16** only reduced it to 58%.[18] This *N*-acetyl derivative is the major urinary metabolite of 1 in humans.[19] The minor metabolites[20] **6** and *N*-formylaminoglutethimide [**17**, 3-ethyl-3-[4-(formylamino)phenyl]piperidine-2,6-dione] were only weakly inhibitory. Therefore, the metabolism of 1 is adverse inasmuch as it leads to less active or inactive metabolites. In addition, these results and the results for the other compounds (**18** and **19**) lacking an amino group afforded further evidence that the presence of an amino group may be a requirement for inhibitory action. It is possible (see below) that the adverse influence of the additional hydroxy substituent in the aminohydroxy derivatives (**20** and **21**) related to the decreased lipophilicity thus conferred.

In a second group of analogues, the effect of relocating the amino group was explored. Relocation of the amino group had a profound effect on the inhibition of desmolase (Figure 1) and aromatase (Figure 2). Thus, **4** was a desmolase inhibitor but had no activity toward aromatase. Similarly, **2** was a strong desmolase inhibitor, with only a weak inhibitory action in the aromatase assay. The remaining isomer, **5**, inhibited neither enzyme system. Values of apparent K_i were determined for the analogues

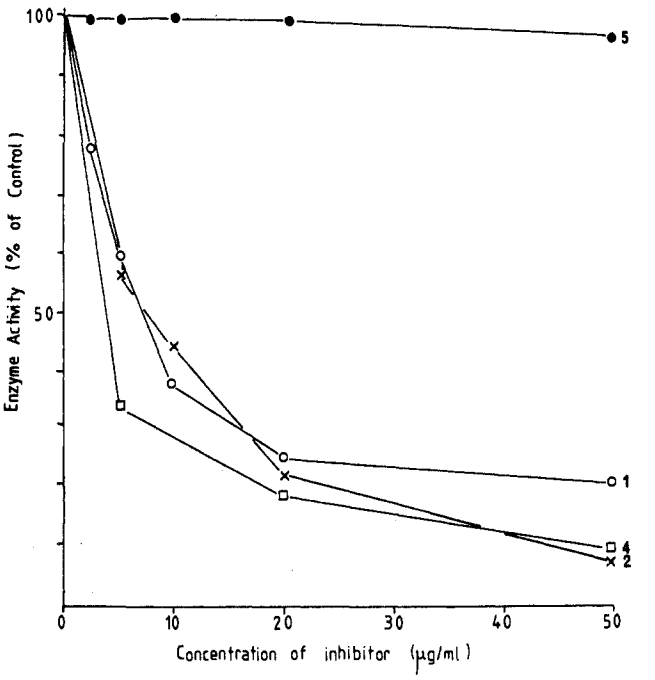

Figure 1. Inhibitory effect of aminoglutethimide (1), its meta isomer (2), *N*-aminoglutethimide (4), and 5-aminoglutethimide (5) toward desmolase from bovine adrenals.

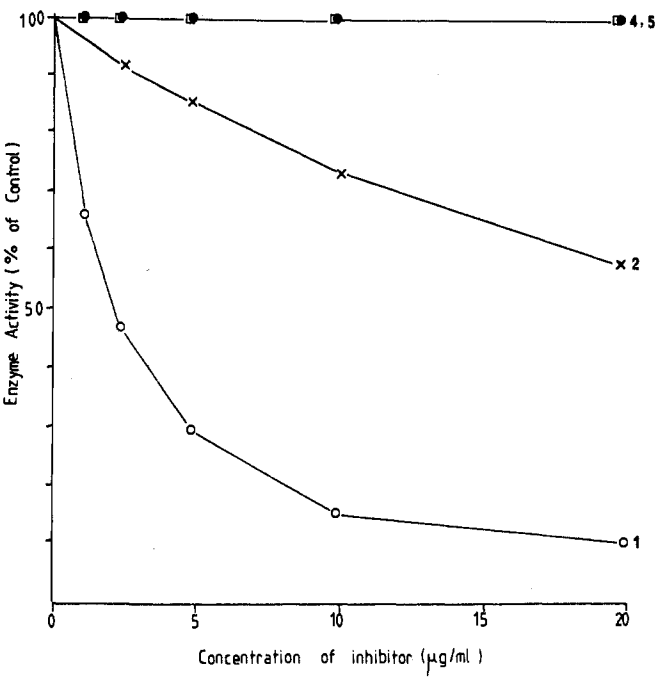

Figure 2. Inhibitory effect of aminoglutethimide (1), its meta isomer (2), *N*-aminoglutethimide (4), and 5-aminoglutethimide (5) toward aromatase from human placenta.

exhibiting effective inhibition (Table II). As well as confirming the previous finding[13] that 4 was a better inhibitor of desmolase than 1, the present studies have identified it as selectively inhibitory toward this enzyme, inasmuch as it was inactive against aromatase. The relative potencies of these three effective inhibitors of desmolase (1, 2, and 4) accorded well with their efficacies as inhibitors of corticosteroid production by bovine adrenal cortex tissue in vitro.[21] Thus, 4 was completely inhibitory at a concentration of 6 μg/mL compared with 30 μg/mL for 1 and 2. However, in other tests,[22] 2 was less potent

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Table III. Extent of Inhibition of Desmolase and Aromatase of Diamino Derivatives of Glutethimide Compared with Aminoglutethimide

no.	compd	% inhibn of desmolase (inhibitor concn 50 μg/mL)	% inhibn of aromatase (inhibitor concn 20 μg/mL)
1	*p*-amino-glutethimide	85	90
14	*N*-amino-*p*-amino-glutethimide	50	32
10	5-amino-*p*-amino-glutethimide	45	21

than 1. Thus, 25 mg/kg produced less inhibition both of cortisol secretion in dogs (46 vs. 66%) and of corticosteroid secretion in rats (40 vs. 81%).

Finally, two analogues containing a second amino group were examined (Table III). The effect of the second amino substituent was adverse, since neither disubstituted derivative (10 or 14) was as inhibitory in either assay as was 1. The results augmented the evidence (see Table I, compounds 20 and 21) that a second substituent that reduced the lipophilicity of the molecule compared with 1 was likely to diminish inhibitory activity toward these enzymes.

In summary, this relatively limited structure–activity study has revealed 4 as selectively inhibitory toward desmolase and more strongly inhibitory than 1, and 2 as markedly selective for desmolase and as inhibitory as 1. The activity of 2 and 4 and 1 against hormone-dependent mammary tumors in rodents is being studied, since it may help to clarify the role of desmolase inhibition as a contributor to the tumor-inhibitory action of 1.

Experimental Section

^{1}H NMR spectra (60 MHz) were obtained with a Perkin-Elmer R12B spectrometer. Mass spectra were determined with a VG 7070H spectrometer and VG 2235 data system, using the direct-insertion method. Electron-impact spectra ($M^{+\cdot}$ ions) were determined at an ionizing voltage of 70 eV, and chemical-ionization mass spectra [$(M + H)^+$ ions] were determined at 50 eV with methane as reagent gas. For thin-layer and column chromatography, Merck Kieselgel 60 was used with the specified Art. numbers: 7730 for TLC (UV detection with a Hanovia Chromatolite), 7734 for conventional column chromatography, 9385 for flash chromatography,[23] and 1511 for preparative HPLC with initial concentration of the sample onto 7734 and using a Jobin-Yvon Chromatospac Prep 10 coupled to a Cecil 212A UV monitor set at 250 nm and a Kelvin Servoscribe recorder. GLC was performed on a Pye 204 instrument using the conditions given below. Melting points were determined on a Kofler hot-stage or a Gallenkamp apparatus and are uncorrected.

Glutethimide (11) and aminoglutethimide (1) were gifts from Ciba-Geigy Ltd. (Horsham, U.K.), who also provided a sample of *m*-aminoglutethimide (2) for comparison purposes. The following compounds were prepared by published procedures: 5-amino-3-ethyl-3-phenylpiperidine-2,6-dione (5),[14] 3-[4-(acetylamino)phenyl]-3-ethylpiperidine-2,6-dione (16, *N*-acetylaminoglutethimide),[14] 3-ethyl-3-[4-(formylamino)phenyl]-3-ethylpiperidine-2,6-dione (17, *N*-formylaminoglutethimide),[20] 3-ethyl-3-(4-hydroxyphenyl)piperidine-2,6-dione (18, *p*-hydroxyglutethimide),[14] and 3-ethyl-3-(4-hydroxy-3-nitrophenyl)-piperidine-2,6-dione (19, *p*-hydroxy-*m*-nitroglutethimide).[24]

For the biochemical studies, D-glucose 6-phosphate, NADP, and glucose-6-phosphate dehydrogenase were purchased from Boehringer-Mannheim. [26-^{14}C]Cholesterol and [1β,2β-^{3}H]testosterone were from Amersham International, U.K. All reagents and solvents were of AR grade. Centrifugations were performed on a MSE 6L centrifuge and MSE Europa 50 ultracentrifuge. Radioactive samples were counted on a Packard Tri-Carb Model 3380 scintillation counter, using Fisofluor "1" (Fisons Scientific Apparatus, Loughborough, U.K.) as the scintillation medium.

Nitration of Glutethimide. Glutethimide (11; 5 g, 0.023 mol) was treated with boiling HNO_3 (70%, 55 mL) for 1 h. The resulting white solid [4.6 g, mixture of 6, 12, and 13; 60:24:16 by GLC (see below)] was fractionally crystallized to yield, successively, *p*-nitroglutethimide (6; 0.55 g, from EtOH), mp 142–143 °C (lit.[15] 140–141 °C); *o*-nitroglutethimide (13; 0.42 g, from MeOH), mp 213.5–214.5 °C (lit.[15] 217–219 °C); and *m*-nitroglutethimide (12; 0.2 g, from MeOH), mp 174–175 °C (lit.[15] 172–174 °C).

Gas–Liquid Chromatography (GLC) of Nitroglutethimide. A solution containing 1 mg in CH_3CN (1 mL) and MeI (1 mL) containing freshly prepared Ag_2O (0.05 g) was heated under reflux for 1 h. The concentrated supernatant was dissolved in EtOAc (1 mL), and aliquots (1 μL) were injected into the gas chromatograph, which was fitted with a flame-ionization detector and a glass column (1.5 m × 4 mm i.d.) packed with 3% OV-1 on Gas chrom Q (100–120 mesh) and operating at an injector temperature of 250 °C, a column temperature of 250 °C, and a N_2 flow rate of 29 mL/min. The retention times of the N-methylated derivatives of 6, 12, and 13 (structures confirmed by mass spectrometry) were, respectively, 2.3, 2.8, and 3.25 min.

3-(3-Aminophenyl)-3-ethylpiperidine-2,6-dione (2, *m*-Aminoglutethimide). A solution of *m*-nitroglutethimide (12; 0.27 g, 0.00102 mol) in dioxane (1 mL, dried over Na) and cyclohexene (20 mL) containing 10% Pd/C (0.12 g) was heated under reflux for 3 h (catalytic transfer reduction[25] gave fewer byproducts than did conventional hydrogenation in our hands). Flash column chromatography (15 × 3 cm column; $CHCl_3$–MeOH, 19:1) afforded 2 (0.16 g, 67%) as colorless crystals from *i*-PrOH: mp 79.5–86.5 °C (polymorphism: lit.[14] 175–178 °C; 117–119 °C for a sample provided by Ciba-Geigy Ltd.) Anal. ($C_{13}H_{16}N_2O_2$) C, H, N.

5-Amino-3-ethyl-3-(4-nitrophenyl)pyridine-2,6-(3*H*,4*H*)-dione (8). Bromination (for procedure, see ref 26) of *p*-nitroglutethimide (6; 3.5 g, 0.013 mol) afforded crude 5-bromo-3-ethyl-3-(4-nitrophenyl)piperidine-2,6-dione (7; oil; 3.2 g) [NMR ($CDCl_3$) δ 0.92 (t, 3 H, CH_3CH_2), 1.8–2.4 (m, 2 H, CH_3CH_2), 2.7–3.0 (m, 2 H, H-4), 4.45 (d × d, 1 H, H-5), 7.52 and 8.30 (AB q, 4 H, Ar H)], which was used without purification for the next stage. A mixture of 7 (2.86 g) and sodium azide (1.68 g) in H_2O (2.1 mL) and dimethyl sulfoxide (8.4 mL) was heated at 100 °C for 2 h and then diluted with H_2O. After extraction with CH_2Cl_2, 8 (0.6 g, 18% based on 6) was obtained as pale yellow rosettes from EtOH: mp 169.5–172 °C; mass spectrum, *m/z* 275 ($M^{+\cdot}$). Anal. ($C_{13}H_{13}N_3O_4$) C, H, N.

5-Amino-3-(4-aminophenyl)-3-ethylpiperidine-2,6-dione (10). A solution of 8 (0.315 g, 0.00115 mol) in EtOH (6.5 mL) containing 10% Pd/C (0.035 g) was stirred under H_2 at 1 atm for 70 h at room temperature. Chromatography (30 × 1 cm column, $CHCl_3$–MeOH, 19:1) afforded first 5-amino-3-(4-aminophenyl)-3-ethylpyridine-2,6(3*H*,4*H*)-dione (9) obtained as yellow crystals (0.89 g, 31%) from toluene: mp 52–65 °C; mass spectrum, *m/z* 245 ($M^{+\cdot}$). Further elution afforded 10, which gave white crystals (0.109 g, 39%) from toluene: mp 61–64 °C; mass spectrum, *m/z* 247 ($M^{+\cdot}$); NMR ($CHCl_3$) δ 0.82 (t, 3 H, CH_3CH_2), 1.7–2.75 (m, 4 H, CH_3CH_2, H-4), 3.45 (d × d, 1 H, H-5), 6.62 and 7.06 (AB q, 4 H, Ar H). Anal. ($C_{13}H_{17}N_3O_2$) C, H, N.

4-Phenyl-4-carboxamidohexanoic Acid Hydrazide (15). Glutethimide (11; 5 g, 0.023 mol) dissolved with stirring in hydrazine hydrate (50 mL) within 5 min at room temperature. After 10 min, white crystals appeared, which after 18 h were collected and washed with further hydrazine hydrate (50 mL), which was removed during 3 days over $CaCl_2$ under vacuum to yield 15 (4.46 g, 78%): mp 131–132 °C; mass spectrum, *m/z* 249 ($M^{+\cdot}$). Anal. ($C_{13}H_{19}N_3O_2$) C, H, N.

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1-Amino-3-ethyl-3-phenylpiperidine-2,6-dione (4, *N*-Aminoglutethimide). A mixture of **15** (2 g, 0.00803 mol) and xylene (60 mL) was heated under reflux during 3 h. Solvent was removed, and the residue was treated with $CHCl_3$. Elution from a flash column (15 × 3 cm) of silica gel with $CHCl_3$ afforded **4** (oil, 0.9 g, 48%), which slowly solidified to colorless crystals: mp 73–74 °C (lit.[16] 72–73 °C); mass spectrum, m/z 232 ($M^{+\cdot}$); NMR (CCl_4) δ 0.85 (t, 3 H, CH_3CH_2), 1.7–2.7 (m, 6 H, CH_3CH_2, H-5, H-4), 5.15 (br s, 2 H, N-NH_2), 7.2 (br s, 5 H, Ar H).

1-Amino-3-(4-aminophenyl)-3-ethylpiperidine-2,6-dione (14). Aminoglutethimide (1; 2.7 g, 0.0116 mol) was heated in a sealed tube at 150 °C with 1 M NaOH (10 mL) for 3 days. The cooled solution was adjusted to pH 3 (1 M HCl) and extracted with EtOAc (4 × 50 mL). The aqueous phase was saturated with KH_2PO_4 and again extracted. The combined extracts (1.7 g) were virtually homogeneous on TLC ($CHCl_3$–MeOH–HOAc, 85:15:5), R_f 0.35; the mass spectrum ($M^{+\cdot}$ at m/z 251) was consistent with the structure 2-(4-aminophenyl)-2-ethylglutaric acid (**22**): NMR (CD_3OD) δ 0.85 (t, 3 H CH_3CH_2), 2.05 (q, 2 H, CH_3CH_2), 2.15 (m, 4 H, H-3, H-4), 6.70 and 7.10 (AB q, 4 H, Ar H)].

Pyrolysis of the hydrazinium salt of **22** (1.25 g, 0.005 mol) (for procedure, see ref 16) and preparative HPLC (silica gel; 150 g) of the crude product (1.27 g), eluting with $CHCl_3$–MeOH, 19:1, at 60 mL/min, afforded **14** (0.14 g, 11%) as a colorless oil: mass spectrum, m/z 248 [$(M + H)^+$]; NMR ($CDCl_3$) δ 0.93 (t, 3 H, CH_3CH_2), 1.86–2.88 (m, 6 H, CH_3CH_2, H-4, H-5), 4.38 (br s, 4 H, N-NH_2, Ar NH_2), 6.74 and 7.11 (AB q, 4 H, Ar H). A solution of **14** (0.056 g) and picric acid (0.2 g) in EtOH (8 mL) was concentrated to ~4 mL, whereupon **14** picrate (0.091 g) separated as a fine yellow powder: mp 195 °C dec. Anal. ($C_{19}H_{20}N_6O_9$) C, H, N.

3-(4-Amino-3-hydroxyphenyl)-3-ethylpiperidine-2,6-dione (20, *p*-Amino-*m*-hydroxyglutethimide). The synthesis of **20** from 1 was based on a published procedure[27] for converting aromatic amines into their hydroxy derivatives via the corresponding *O*-sulfates. Aminoglutethimide (1; 0.232 g, 0.001 mol) dissolved during 1 h in stirred aqueous KOH (2 M, 2 mL). Potassium persulfate (0.27 g, 0.001 mol) was added, and after a further 16 h, the dark red solution was heated under reflux with 5 M HCl (1 mL) for 1 h, diluted with H_2O (10 mL), neutralized with $NaHCO_3$, and then extracted with CH_2Cl_2 (50 mL) and EtOAc (3 × 50 mL). Preparative TLC (2-mm layer, 20 × 20 cm plate) in $CHCl_3$–MeOH (9:1) separated 1 (R_f 0.50) from the product (R_f 0.35), which was eluted with $CHCl_3$–MeOH (3:1, 50 mL) and crystallized from hot H_2O after decolorizing (charcoal) to afford the monohydrate of **20** as colorless prisms (0.0145 g, 6%): mp 105–107 °C; mass spectrum, m/z 248 ($M^{+\cdot}$). Anal. ($C_{13}H_{18}N_2O_4$) C, H, N.

3-(3-Amino-4-hydroxyphenyl)-3-ethylpiperidine-2,6-dione (21, *m*-Amino-*p*-hydroxyglutethimide). A solution of 3-ethyl-3-(4-hydroxy-3-nitrophenyl)piperidine-2,6-dione[24] (**19**; 0.14 g, 0.0005 mol) in EtOH (25 mL) containing PtO_2 (0.1 g) was stirred under H_2 at 1 atm until uptake was complete. Compound **21** (0.080 g, 64%) crystallized from EtOH–toluene: mp 162–164 °C; mass spectrum, m/z 248 ($M^{+\cdot}$); NMR (CD_3OD) δ 0.96 (t, 3 H, CH_3CH_2), 1.8–2.8 (m, 6 H, CH_3CH_2, H-4, H-5), 6.45–7.1 (m, 3 H, Ar H). Anal. ($C_{13}H_{16}N_2O_3$) C, H, N.

Enzyme Preparation and Assay Procedures. The following applies to both assay procedures. Preliminary studies were performed to determine the possible inhibitory effects of the compounds. These screening assays were run in duplicate, and the amount of product formed was averaged. Each compound was examined over a range of concentrations. At each concentration of inhibitor, samples were removed from each assay tube at three time points. The results were plotted on a graph of product released against time of incubation. The resulting linear graph was utilized to determine the rate of enzyme reaction at each concentration of inhibitor. The values were then compared to control samples (no inhibitor present) run simultaneously and are reported as percent activity of control samples.

Compounds exhibiting effective inhibition in the screening assays were evaluated further by determination of the apparent K_i values. The apparent K_m for the substrates and the apparent K_i were determined from Lineweaver–Burk plots, using the method of least squares analysis to obtain a linear fit to the data.

A. Desmolase. The mitochondrial fraction of bovine adrenal cortex provided the source of desmolase,[25] and the method of isolation was essentially as published. After resuspension in the minimum volume of buffered sucrose, the isolated mitochondria were stored at −70 °C. Protein concentration was determined by the method of Lowry, as modified by Hartree.[28] Enzyme activity remained stable for periods of 2–3 months.

We assayed the enzyme activity using [26-^{14}C]cholesterol as substrate and measuring the [^{14}C]isocaproic acid released.[29,30] Inhibitor compounds were added in ethanol (10 μL), and an equal volume of ethanol was added to the control assay. Total volume of the assay mixture was 1.0 mL. After preincubating the flasks at 30 °C for 5 min, we started the reaction by the addition of 1 mg of mitochondrial protein. Samples were removed after 2, 4, and 6 min of incubation, and the assay was completed as described.[29] The enzymatic rate of reaction was measured as picomoles of cholesterol cleaved per minute per milligram of protein.

B. Aromatase. Aromatase was obtained from the microsomal fraction of human placental tissue.[31] The isolated microsomes were suspended in the minimum volume of 50 mM phosphate buffer (pH 7.4) and stored at −30 °C. Protein concentration was assayed as described above. Enzyme samples showed no loss of activity after 3–4 months.

We monitored activity by measuring the ^3H_2O formed from [1β,2β-^{3}H]testosterone during aromatization.[32] The assay procedure was as described, except that the substrate concentration was 1.5 μM, and after preincubation of the tubes at 30°C for 5 min, the reaction was started by the addition of 0.5 mg of microsomal protein. Samples were taken after 5, 10, and 15 min of incubation. The enzymatic rate was measured as milligrams of testosterone aromatized per minute per milligram of protein.

Acknowledgment. This investigation was supported by a grant from the Medical Research Council and the Cancer Research Campaign. We thank M. H. Baker for skilled technical assistance.

Registry No. 1, 125-84-8; **2**, 83417-11-2; 4, 4238-75-9; **5**, 56392-80-4; **6**, 38527-73-0; **7**, 83417-12-3; 8, 83417-13-4; **9**, 83417-14-5; **10**, 83417-15-6; 11, 77-21-4; **12**, 73252-00-3; **13**, 38527-74-1; **14**, 83417-16-7; 14·picrate, 83417-18-9; **15**, 83435-77-2; **19**, 83417-21-4; **20**, 83417-19-0; **21**, 83417-20-3; **22**, 83417-17-8; aromatase, 9039-48-9.

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