Analogues of Aminoglutethimide Based on 1-Phenyl-3-azabicyclo[3.1.0]hexane-2,4-dione: Selective Inhibition of Aromatase Activity

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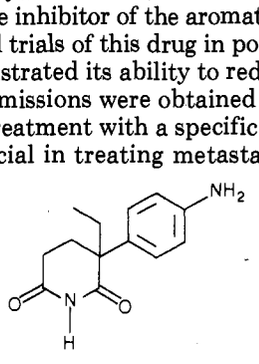
In exploring the structural features responsible for the inhibitory activity of aminoglutethimide [3-(4-aminophenyl)-3-ethylpiperidine-2,6-dione] (1) toward the cholesterol side chain cleavage (CSCC) enzyme from bovine adrenals and the human placental aromatase enzyme, analogues have been synthesized in which the piperidine-2,6-dione ring is replaced by substituted or unsubstituted azabicyclo[3.1.0]hexane-2,4-dione rings. The unsubstituted analogue 1-(4-aminophenyl)-3-azabicyclo[3.1.0]hexane-2,4-dione (**9a**) is a slightly more potent inhibitor of aromatase than 1 (K_i = 1.2 μM, cf. 1.8 μM for 1) but is noninhibitory toward the CSCC enzyme. The substituted analogues 1-(4-aminophenyl)-3-butyl-3-azabicyclo[3.1.0]hexane-2,4-dione (**9e**) and 1-(4-aminophenyl)-3-pentyl-3-azabicyclo-[3.1.0]hexane-2,4-dione (**9f**) are approximately 100 times more potent than 1 (K_i values of **1**, **9e**, and **9f** are 1.8, 0.015, and 0.02 μM, respectively) in inhibiting aromatase, with no significant activity toward the CSCC enzyme. Type II difference spectra were exhibited by **1**, **9a**, and **9f** in their interaction with the aromatase enzyme (respective K_s values of **1**, **9a**, and **9f** are 0.13, 0.08, and 0.01 μM). Modification of the para amino function by alkylation, its relocation, replacement by H, or replacement by a methyl, aldehyde, or secondary alcohol group produced analogues that were inactive toward both enzyme systems.

Aminoglutethimide [3-(4-aminophenyl)-3-ethylpiperidine-2,6-dione] (1) is an effective endocrine therapy for metastatic breast cancer in postmenopausal women.[1,2] The drug acts by reducing circulating estrogen levels, thereby removing the stimulus required for the growth of the hormone-dependent tumors. This action is mediated via inhibition of several cytochrome P450 dependent steroidogenic enzymes. Its most potent activity is toward the aromatase enzyme, which is responsible for the conversion of the androgens androstenedione and testosterone into estrone and estradiol, respectively.[3] The other major action is against the initial step in steroid hormone biosynthesis, namely the conversion of cholesterol to pregnenolone by the cholesterol side chain cleavage (CSCC) enzyme.[4] Inhibition of the CSCC enzyme reduces adrenal corticosteroid production, so replacement hydrocortisone therapy is required to prevent the reflex rise in ACTH, which would overcome the blockade of the CSCC enzyme. Therefore, a potent selective inhibitor of aromatase activity would be of therapeutic advantage. The steroid 4-hydroxyandrostenedione is a potent, specific, and irreversible inhibitor of the aromatase enzyme.[5,6] Preliminary clinical trials of this drug in postmenopausal patients have demonstrated its ability to reduce plasma estradiol levels, and remissions were obtained in some patients.[7] Therefore, treatment with a specific aromatase inhibitor can be beneficial in treating metastatic breast cancer.



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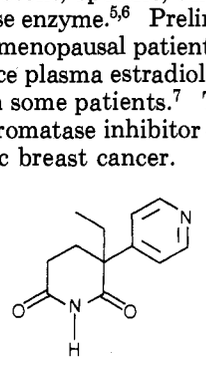

1a

The present study concerns the development of analogues of aminoglutethimide that selectively inhibit aromatization. Previous investigations were concerned with the introduction of an additional amino group into the molecule and relocation of the amino function; although the *N*-amino analogue of 1 was identified as a selective inhibitor of the CSCC enzyme, no leads to a pure aromatase inhibitor emerged.[8] These and other studies[9] confirmed that only basic molecules displayed inhibitory activity toward aromatase.

Subsequently, the evaluation of derivatives of 1 containing stronger basic substituents than aniline enabled the 4-pyridyl analogue **1a** to be identified as a selective aromatase inhibitor,[10] although it was not as active as 1. However, the potency of the 4-pyridyl analogue was enhanced approximately 10-fold by the addition of 1-alkyl and 3-alkyl substituents.[11] Similarly, 3-alkyl analogues of 1 are more potent aromatase inhibitors but retain activity toward the CSCC enzyme.[12] Replacement of the six-membered piperidine-2,6-dione (glutarimide) ring of 1 with a five-membered pyrrolidine-2,5-dione ring produced compounds with no activity toward the CSCC enzyme but retaining the aromatase inhibitory capability of 1.[13] Therefore, the objective remains of obtaining an analogue of 1 that inhibits aromatase selectively and with greatly increased potency. This present paper describes the synthesis and evaluation of analogues of aminoglut-

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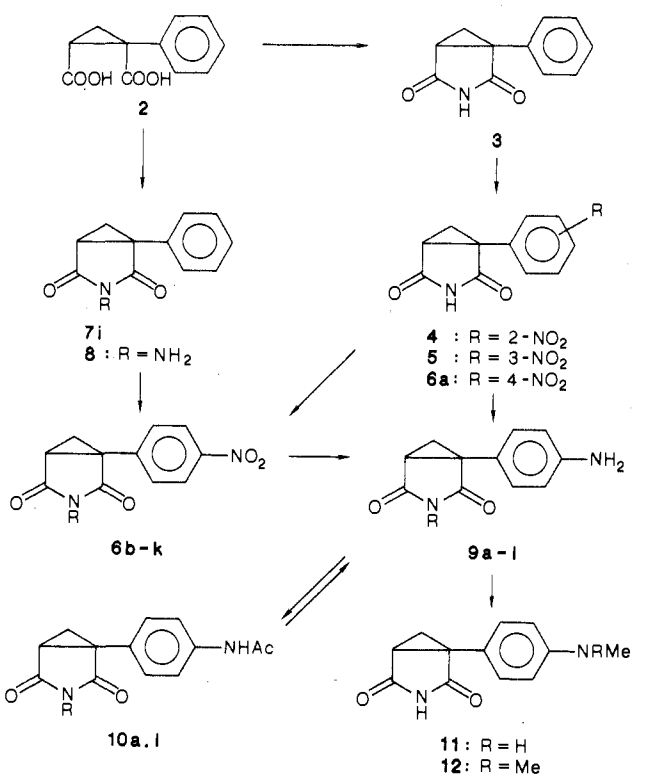
Scheme I[a]

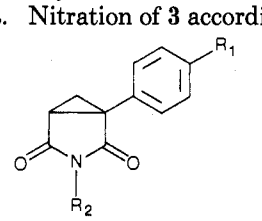


[a] a: R = H; **b**: R = CH_3; **c**: R = C_2H_5; **d**: R = C_3H_7; e: R = C_4H_9; **f**: R = C_5H_{11}; **g**: R = $CH(CH_3)_2$; **h**: R = $CH_2CH(CH_3)_2$; i: R = neopentyl; **j**: R = $CH_2C{\equiv}CH$; **k**: R = benzyl; **l**: R = $CH_2CH{=}CH_2$.

ethimide in which the glutarimide ring has been replaced by an azabicyclohexanedione ring to produce a series of selective, highly potent aromatase inhibitors.

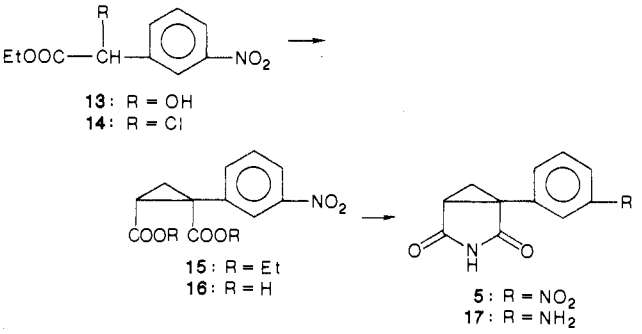
Results and Discussion

Synthesis of Analogues. The amino-substituted 1-phenyl-1,2-cyclopropanedicarboximide analogues of **1** (see Table I) were synthesized (Scheme I) with 1-phenylcyclopropanedicarboxylic acid **2**[14,15] or the imide **3**[15] as starting materials. Nitration of **3** according to Hoffmann



9a: $R_1 = NH_2$; $R_2 = H$
9b: $R_1 = NH_2$; $R_2 = CH_3$
9c: $R_1 = NH_2$; $R_2 = C_2H_5$
9d: $R_1 = NH_2$; $R_2 = C_3H_7$
9e: $R_1 = NH_2$; $R_2 = C_4H_9$
9f: $R_1 = NH_2$; $R_2 = C_5H_{11}$
9g: $R_1 = NH_2$; $R_2 = CH(CH_3)_2$
9h: $R_1 = NH_2$; $R_2 = CH_2CH(CH_3)_2$
9i: $R_1 = NH_2$; $R_2 = CH_2C(CH_3)_3$
9j: $R_1 = NH_2$; $R_2 = CH_2C{\equiv}CH$
9k: $R_1 = NH_2$; $R_2 = CH_2C_6H_5$
9l: $R_1 = NH_2$; $R_2 = CH_2CH{=}CH_2$
3 : $R_1 = H$; $R_2 = H$
8 : $R_1 = H$; $R_2 = NH_2$
11: $R_1 = NHCH_3$; $R_2 = H$
12: $R_1 = N(CH_3)_2$; $R_2 = H$
19: $R_1 = CH_3$; $R_2 = CH_2CH_2OH$
24: $R_1 = CH_2OH$; $R_2 = CH_3$
25: $R_1 = CH{=}O$; $R_2 = CH_3$

Scheme II



and Urech[16] gave a mixture of isomers, from which the para nitro derivative **6a**[17] was obtained in pure form by crystallization. From the mother liquors of **6a**, the ortho nitro derivative **4** and a fraction rich in **5** were also isolated by preparative chromatography. All three isomers, **4–6a**, were identified by ^1H NMR; the yields were 57% of **6a** and 4.6% of **4**. The meta derivative **5** was additionally synthesized by an independent route (Scheme II) with meta nitromandelic acid ethyl ester **13**[18] as starting material and with dicarboxylic acid **16** as intermediate.

The first aminoglutethimide analogues were formed by catalytic hydrogenation of **6a** and **5**; interestingly, **9a**[19] inhibited the aromatase reaction slightly more potently than aminoglutethimide and had no effect on CSCC.

The N-substituted imides **6b–k** were prepared by alkylation of **6a** with alkyl halides in the presence of sodium hydride. In individual cases, instead of alkylation of **6a** with slow-reacting alkyl halides, conversion of the anhydride formed in situ from **2** with, e.g., neopentylamine, followed by cyclization and then nitration to **6g** proved a more efficient route. By the above-mentioned, slightly modified method of Taub,[20] the *N*-amino imide **8** analogue of *N*-aminodoriden[8] was likewise synthesized.

Catalytic hydrogenation of **6a–i** and **6k**, or reduction of **6j** with metallic tin in hydrochloric acid, led to **9a–k**. The corresponding *N*-allyl derivative **9l** was synthesized by alkylation of the *N*-acetate **10a** and subsequent solvolysis of the *N*-acetyl protecting group.

Starting from **9a**, the *N*-methyl imide **9b** was obtained directly by alkylation with diazomethane and the N-methylated anilines **11** and **12** by way of an analogous reaction with dimethyl sulfate.

The synthesis of some phenylcyclopropanedicarboximides without basic substitution is summarized in Scheme III. As above, upon condensation with ethanolamine, the anhydride formed in situ from **18** yielded the imide **19**. The *N*-methyl derivative **21** was brominated on the side chain of the paratolyl group with *N*-bromosuccinimide to give the crystalline monobromo derivative **22**. This was, according to the elemental analysis (Br: calcd, 27.17; found, 28.4), contaminated with the corresponding dibromide **23**. Without further purification, this mixture was hydrolyzed in THF/water and gave finally the oxygen-containing derivatives **24** and **25**.

Enzyme Activity of Analogues. Table II compares the inhibitory potency of aminoglutethimide toward the aromatase and CSCC enzyme systems with that of aza-

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Table I. Physical Properties of 1-Phenylcyclopropanedicarboximides

compd	R_1	R_2	mp, °C	recryst solvent	yield, % (procedure)[a]	formula
3	H	H	137–138	EtOAc	89 (A)	$C_{11}H_9NO_2$
4	2-NO_2	H	169	EtOAc/PE[d]	4.6 (B)	$C_{11}H_8N_2O_4$
5	3-NO_2	H	173–174	EtOAc/PE	98 (A)	$C_{11}H_8N_2O_4$
			150–158	EtOAc/PE	22 (B)[b]	
6a	4-NO_2	H	174–175	EtOAc	57 (B)	$C_{11}H_8N_2O_4$
6b	4-NO_2	CH_3	148–150	EtOAc/PE	69 (C)	$C_{12}H_{10}N_2O_4$
6c	4-NO_2	C_2H_5	155–157	EtOAc/PE	81 (C)	$C_{13}H_{12}N_2O_4$
6d	4-NO_2	C_3H_7	97	Et_2O	86.5 (C)	$C_{14}H_{14}N_2O_4$
6e	4-NO_2	C_4H_9	oil		73 (C)	$C_{15}H_{16}N_2O_4$
6f	4-NO_2	C_5H_{11}	oil		90 (C)	$C_{16}H_{18}N_2O_4$
6g	4-NO_2	$CHMe_2$	115–118	EtOAc/PE	64 (C)	$C_{14}H_{14}N_2O_4$
6h	4-NO_2	CH_2CHMe_2	100–101	EtOAc/PE	56 (C)	$C_{15}H_{16}N_2O_4$
6i	4-NO_2	CH_2CMe_3	118–119	Et_2O/PE	16 (C)	$C_{16}H_{18}N_2O_4$
6j	4-NO_2	$CH_2C{\equiv}CH$	163–165	EtOAc/PE	70 (C)	$C_{14}H_{10}N_2O_4$
6k	4-NO_2	benzyl	147–149	EtOAc	69.7 (C)	$C_{18}H_{14}N_2O_4$
7i	H	CH_2CMe_3	123	EtOH	66.8 (D)	$C_{16}H_{19}NO_2$
8	H	NH_2	oil		18 (D)	$C_{11}H_{10}N_2O_2$
9a	4-NH_2	H	183–185	EtOH	85.2 (E)	$C_{11}H_{10}N_2O_2$
9b	4-NH_2	CH_3	137–139	EtOAc/PE	85 (E)	$C_{12}H_{12}N_2O_2$
9c	4-NH_2	C_2H_5	116–118	EtOAc/PE	77 (E)	$C_{13}H_{14}N_2O_2$
9d	4-NH_2	C_3H_7	114–115	EtOAc/PE	77.5 (E)	$C_{14}H_{16}N_2O_2$
9e	4-NH_2	C_4H_9	95–98	Et_2O/PE	78 (E)	$C_{15}H_{18}N_2O_2$
9f	4-NH_2	C_5H_{11}	113–115	EtOAc/PE	80 (E)	$C_{16}H_{20}N_2O_2$
9g	4-NH_2	$CHMe_2$	160–163	EtOAc/PE	76 (E)	$C_{14}H_{16}N_2O_2$
9h	4-NH_2	CH_2CHMe_2	115–117	Et_2O/PE	53.6 (E)	$C_{15}H_{18}N_2O_2$
9i	4-NH_2	CH_2CMe_3	140–143	Et_2O/PE	78.7 (E)	$C_{16}H_{20}N_2O_2$
9j	4-NH_2	$CH_2C{\equiv}CH$	106–109	EtOAc/PE	32	$C_{14}H_{12}N_2O_2$
9k	4-NH_2	benzyl	154–155	EtOH	68.5 (E)	$C_{18}H_{16}N_2O_2$
9l	4-NH_2	$CH_2CH{=}CH_2$	104–106	EtOAc/PE	48	$C_{14}H_{14}N_2O_2$
10a	4-NHAc	H	>230	EtOH	77	$C_{13}H_{12}N_2O_3$
10l	4-NHAc	$CH_2CH{=}CH_2$	113–116	EtOAc/PE	63	$C_{16}H_{16}N_2O_3$
11	4-$NHCH_3$	H	192–194	EtOH	48	$C_{12}H_{12}N_2O_2$
12	4-$N(CH_3)_2$	H	190–191	EtOAc/PE	5	$C_{13}H_{14}N_2O_2$
17	3-NH_2	H	135–137	EtOAc/PE	48 (E)	$C_{11}H_{10}N_2O_2$
19	4-CH_3	$(CH_2)_2OH$	96–97	EtOAc	70.3 (D)	$C_{14}H_{15}NO_3$
20	4-CH_3	H	118	EtOAc	76 (A)	$C_{12}H_{11}NO_2$
21	4-CH_3	CH_3	73–74	Et_2O/PE	51 (C)	$C_{13}H_{13}NO_2$
22	4-CH_2Br	CH_3	94–96	Et_2O/PE	77.8	$C_{13}H_{12}BrNO_2$[c]
24	4-CH_2OH	CH_3	168–169	EtOAc/PE	47	$C_{13}H_{13}NO_3$
25	4-COH	CH_3	168–169	EtOAc	6	$C_{13}H_{11}NO_3$

[a] See the Experimental Section. [b] According to ^1H NMR, a 4:1 mixture of **5** and **6a**. [c] Anal. Br: calcd, 27.17; found, 28.4. [d] PE = petroleum ether.

bicyclohexanedione analogues possessing an amino group on the para position of the phenyl ring **9a–k**. Unlike **1**, none of these compounds have any significant activity toward the CSCC enzyme. However, all of the analogues inhibit aromatase activity, with either testosterone or androstenedione as substrate. Compound **9a** with an unsubstituted azabicyclohexanedione ring is as potent as aminoglutethimide toward the aromatase enzyme. Substantial increases in potency resulted from the addition of straight chain alkyl groups to the nitrogen atom of the azabicyclohexanedione ring. The most powerful aromatase inhibitors were the analogues with an *n*-butyl (**9e**) or *n*-pentyl (**9f**) group at this position. Branching of the alkyl-chain substituent led to decreased activity (**9g–i**), although these compounds remain more potent than **1**. Similar results were obtained with the propargyl (**9j**), allyl (**9l**), and benzyl (**9k**) compounds.

Structural variations were made to the para amino group on the phenyl ring. Previous studies demonstrated that the *N*-amino analogue of **1** is a potent selective inhibitor of the CSCC enzyme; however, the corresponding *N*-aminoazabicyclohexanedione derivative **8** was inactive in both assays. Inhibition of aromatase was abolished by the loss and reduced by alkylation of the para amino function (**3**, **11**, **12**). In addition, replacement of the amino function with a methyl (**19**), aldehyde (**25**), or hydroxymethyl group (**24**) produced inactive compounds.

The apparent K_i values for the inhibition of the aromatization of androstenedione by **9a,d–f** were determined from competitive type analysis of the Lineweaver–Burk plots (not shown) and compared with that of **1** (Table III). Compounds **9e** and **9f** (K_i values of 0.015 and 0.02 μM, respectively) are approximately 100 times more potent than **1** (K_i = 1.8 μM) in inhibiting the aromatization of androstenedione.

Aminoglutethimide exhibits a type II binding spectrum in its interaction with the cytochrome P450 of human

Table II. Activity of Azabicyclohexanedione Analogues toward the Aromatase and CSCC Enzymes Compared with Aminoglutethimide

	aromatase		
compd	testosterone as substrate: I_{50}, μM	androstenedione as substrate: I_{50}, μM	CSCC: I_{50}, μM
1	8	14	30
9a	7	11	none
9b	2.5	14	460
9c	2.5	7	305
9d	0.2	0.6	328
9e	0.15	0.04	232
9f	0.14	0.085	170
9g	2	4	320
9h	0.2	nd[a]	310
9i	0.44	0.2	294
9j	2	nd	292
9k	2	7	290
9l	1	0.7	274

[a] Nd = not determined.

Scheme III

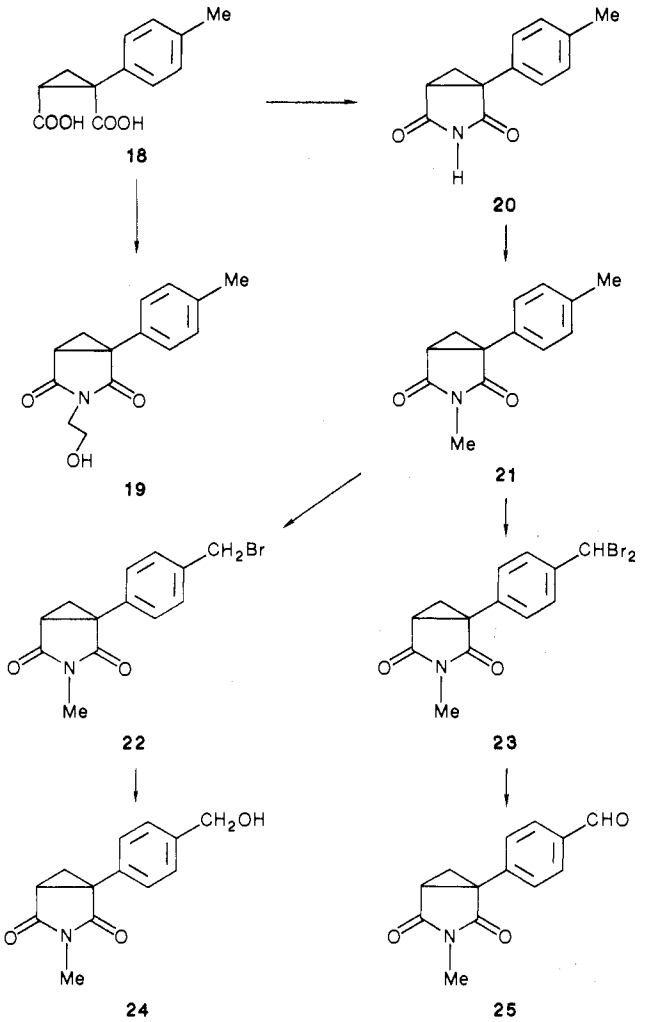

Table III. Apparent K_i and K_s Values for Inhibition of Aromatase by Aminoglutethimide and Azabicyclohexanedione Analogues

compd	apparent K_i,[a] μM	apparent K_s, μM
1	1.8	0.13
9a	1.2	0.08
9d	0.05	0.05
9e	0.0015	nd[b]
9f	0.02	0.01

[a] Androstenedione as substrate. [b] Nd = not determined.

placental microsomes.[21] This interaction is due to the amino group coordinating with the iron atom in the heme of cytochrome P450 and is characterized by an absorbance maximum at 430 nm and a minimum of 408 nm. The requirement for an intact para amino function with the azabicyclohexanedione derivatives suggests that a similar process may be occurring. Three of the analogues were examined for their spectral interactions with placental microsomes and all three were found to elicit a type II binding spectrum, suggesting that the azabicyclohexanedione compounds bind to cytochrome P450 in a similar fashion to 1. Values for the apparent spectral dissociation constants, K_s, produced by **9a**, **9e**, and **9f** are compared with that of 1 in Table III.

This study has identified the azabicyclohexanedione analogues of aminoglutethimide as selective inhibitors of aromatase. The substitution of hydrogen by an alkyl group on the nitrogen atom of the azabicyclohexanedione ring increases the inhibitory activity toward the aromatase enzyme. This may reflect enhanced lipophilicity, enabling the analogues to achieve tighter binding to the active site. However, bulky alkyl substitutions have reduced inhibitory capability, suggesting that a steric requirement may be involved. Further studies with alkyl-substituted analogues of aminoglutethimide and congeners may reveal more about the steric requirements for binding to the aromatase enzyme and enable an improved aromatase inhibitor to be designed for treatment of hormone-dependent breast cancer.

Experimental Section

Melting points were determined in open capillary tubes with a Büchi apparatus according to Dr. Tottoli and are uncorrected. TLC of each compound was performed on Merck F 254 silica gel plates, and column chromatography, if necessary, was performed on Merck silica gel 60 (230–400 mesh). R_f values for characterization of oily compounds were determined with the same solvent systems as by the corresponding preparative chromatography. Elemental analyses were within ±0.4% of theory, except where indicated. The structures of all compounds were confirmed by their IR (Perkin-Elmer 1310 or 298 spectrophotometers) and ^{1}H NMR spectra (Varian HA-100D or Bruker WM-250).

1-Phenyl-3-azabicyclo[3.1.0]hexane-2,4-diones 3, 5, and 20. Method A. According to the procedure of Epstein,[15] a mixture of 0.048 mol of **2**, **16**, or **18** and 5.79 g (0.096 mol) of urea in 300 mL of xylene (isomeric mixture) was boiled for about 16 h under reflux and evaporated to dryness. The residue was dissolved in ethyl acetate, washed with water, dried ($MgSO_4$), and crystallized after removal of the solvent under reduced pressure.

1-(Nitrophenyl)-3-azabicyclo[3.1.0]hexane-2,4-diones 4, 5, and 6a. Method B. A solution of 21.0 g (0.116 mol) of **3** in 28 mL of concentrated sulfuric acid was cooled to −5 °C, 16.8 mL of nitric acid (65%) added within 1.5 h, and the mixture was stirred in an ice bath for 16 h. The reaction mixture was poured onto ice and extracted with methylene chloride. The organic phase was washed with water, dried, and evaporated. The crystalline residue was recrystallized from 200 mL of ethyl acetate, yielding 15.05 g of **6a**.

The mother liquor was evaporated and chromatographed on silica gel with hexane/ethyl acetate, 4:6. The fraction with an R_f of 0.3 yielded 5.7 g of an ca. 4:1 mixture of **5** and **6a**. The fraction with R_f 0.2 yielded 1.2 g of **4**.

1-(3-Nitrophenyl)-3-azabicyclo[3.1.0]hexane-2,4-dione (5). (a) 2-Chloro-2-(3-nitrophenyl)ethyl Acetate. A mixture of 97 g (0.431 mol) of **13**[18] and 69 mL (0.948 mol) of thionyl chloride was stirred for 16 h at room temperature, and a few drops of pyridine were added. After the evolution of gas had ceased, the mixture was stirred for 2 h at 100 °C. Fractional distillation yielded 93.3 g (88.9%) of **14** as a yellowish oil with bp 134 °C/(0.06 mbar). Anal. ($C_{10}H_{10}ClNO_4$) C, H, N.

(b) 1-(3-Nitrophenyl)-1,2-cyclopropanedicarboxylic Acid Diethyl Ester (15). A mixture of 12.2 g (50 mmol) of **14** and 5.45 mL (50 mmol) of acrylic acid ethyl ester was added dropwise to a suspension of 2.2 g (50 mmol) of sodium hydride (55–60% in paraffin oil) in 12 mL of toluene at 50 °C during stirring under nitrogen. To initiate and accelerate the reaction, a few drops of alcohol/ether (1:1) were added occasionally. After the reaction had ended, the mixture was washed with water, dried, and evaporated. The residue was chromatographed on 1 kg of silica gel with CH_2Cl_2 and yielded 5.5 g (35.7%) of **15** as a brown oil, R_f 0.15 (CH_2Cl_2). Anal. ($C_{15}H_{17}NO_6$) C, H, N.

(c) 1-(3-Nitrophenyl)-1,2-cyclopropanedicarboxylic Acid (16). A mixture of 14.2 g (46.3 mmol) **15** in 150 mL of methanol and 100 mL of 1.0 N NaOH was boiled under reflux for 16 h. After removal of the major part of the methanol under vacuum, the residue was acidified with hydrochloric acid and extracted with ether. The organic phase was washed with water, dried, and evaporated. The residue was crystallized from ethyl acetate/petroleum ether and yielded 9.8 g (84.5%) of **16**, mp 167–170 °C. Anal. ($C_{11}H_9NO_6$) C, H, N.

The dicarboxylic acid **16** was converted to **5** by method A, giving a yield of 92%.

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3-Alkyl-1-phenyl-3-azabicyclo[3.1.0]hexane-2,4-diones 6b–k and 21. Method C. Thirty millimoles of alkyl iodide, propargyl bromide, or benzyl chloride was added to a mixture of 4.6 g (20 mmol) of **6a** or **20** and 0.72 g (30 mmol) of sodium hydride (practical grade, Fluka) in 50 mL of DMF, which was then stirred for 5 h. The reaction mixture was evaporated, and the residue was dissolved in ethyl acetate, washed with water, dried, and crystallized after evaporation of the solvent. The oily **6f** was obtained sufficiently pure to be used in the next step; **6e** was chromatographed on 250 g of silica gel with ethyl acetate/petroleum ether (1:2) to give a yellowish oil.

3-Neopentyl-1-phenyl-3-azabicyclo[3.1.0]hexane-2,4-dione (7i). Method D. A mixture of 9.3 g (45 mmol) of **2** in 150 mL of xylene (isomeric mixture) was boiled for 5 h and cooled. Neopentylamine (5.3 mL, 45 mmol) dissolved in 10 mL of xylene was added, and shortly thereafter a mixture of crystalline amide acids precipitated as an intermediate, mp 163 °C.

The reaction mixture was boiled for a further 16 h and evaporated to dryness. The residue was crystallized from ethanol and yielded 7.75 g (66.8%) of **7i**, mp 123 °C.

3-Amino-1-phenyl-3-azabicyclo[3.1.0]hexane-2,4-dione (8). According to a modification of the method of Taub,[20] 20.6 g (0.1 mol) of **2** in 400 mL of xylene (isomeric mixture) was boiled for 5 h and cooled and then, after addition of 5.0 mL (0.1 mol) of hydrazine hydrate, boiled for a further 16 h. The reaction mixture was evaporated, and the residue chromatographed was on silica gel with CH_2Cl_2/MeOH, 5:1. Compound **8** (3.8 g) was obtained as a yellow oil with an R_f of 0.7.

1-(Aminophenyl)-3-azabicyclo[3.1.0]hexane-2,4-diones 9a–i,k and 17. Method E. A 3–5% alcoholic solution of the nitro derivatives **5** or **6a–i,k** was hydrogenated at normal pressure in the presence of 5% Pd/C. After removal of the catalyst, the solvent was distilled off under vacuum, giving products **9a–i,k** or **17**.

1-(4-Aminophenyl)-3-propargyl-3-azabicyclo[3.1.0]hexane-2,4-dione (9j). A mixture of 0.54 g (2 mmol) of **6j** and 1.5 g of powdered tin in 4 mL of water and 4 mL of concentrated HCl was stirred for 1 h at 100 °C. After cooling, the mixture was diluted with 20 mL of water, filtered, made alkaline with NaOH, and extracted with ethyl acetate. The organic phase was washed with water, dried, and evaporated. The residue was recrystallized from ethyl acetate/petroleum ether, yielding 150 mg (32%) of **9j**, mp 106–109 °C.

1-[4-(Acetylamino)phenyl]-3-azabicyclo[3.1.0]hexane-2,4-dione (10). After addition of 6 mL (26 mmol) of acetic anhydride, a solution of 4 g (20 mmol) of **9a** and 120 mg of (dimethylamino)pyridine in 150 mL of THF was left to stand for 16 h at room temperature. The crystalline precipitate was aspirated, washed with THF, and dried to yield 3.75 g (77%), mp >230 °C.

1-[4-(Acetylamino)phenyl]-3-allyl-3-azabicyclo[3.1.0]hexane-2,4-dione (10l). A mixture of 1.2 g (5 mmol) of **10a** and 180 mg (7.5 mmol) of sodium hydride in 25 mL of DMF was alkylated at 0 °C with 0.65 mL (7.5 mmol) of allyl bromide according to method C and worked up. The product was crystallized from ethyl acetate/ether to yield 0.8 g (63%), mp 113–116 °C.

1-(4-Aminophenyl)-3-allyl-3-azabicyclo[3.1.0]hexane-2,4-dione (9l). A mixture of 2.2 g (7.7 mmol) of **10l** in 8 mL of water and 8 mL of concentrated HCl was stirred for 3 h at 100 °C. After cooling, it was diluted with ice–water, made alkaline with sodium hydroxide solution, and extracted with ethyl acetate. The organic phase was washed with water, dried, and concentrated. After addition of petroleum ether, 0.9 g (48%) of **9l** crystallized out, mp 104–106 °C.

1-(4-Aminophenyl)-3-methyl-3-azabicyclo[3.1.0]hexane-2,4-dione (9b). To a solution of 220 mg (1 mmol) of **9a** in 10 mL of methanol was added 5 mL of a 0.3 M ethereal solution of diazomethane at 0 °C. The reaction mixture was left to stand for 16 h and evaporated to dryness. The residue was crystallized from ethyl acetate/petroleum ether and yielded 100 mg of **9b**, mp 136–138 °C.

1-[4-(Methylamino)phenyl]-3-azabicyclo[3.1.0]hexane-2,4-dione (11) and 1-[4-(Dimethylamino)phenyl]-3-azabicyclo[3.1.0]hexane-2,4-dione (12). After addition of 3.8 mL (40 mmol) of dimethyl sulfate, a mixture of 4 g (20 mmol) of **9a** and 5.6 mL (40 mmol) of triethylamine in 120 mL of THF was boiled for 4 h under reflux. After cooling, the reaction mixture was stirred for 30 min with 40 mL of 1.0 N ammonia solution, evaporated, and dissolved in CH_2Cl_2. The solution was washed with water, dried, and evaporated to dryness. The residue was crystallized from ethanol, yielding 1.2 g (27.8%) of **11**, mp 192–194 °C. After evaporation, the product from the mother liquor was chromatographed on silica gel with hexane/ethyl acetate/triethylamine, 50:50:1. The fraction with R_f 0.21 yielded 400 mg of **12**; the fraction with R_f 0.15 yielded a further 350 mg of **11**.

1-[4-(Bromomethyl)phenyl]-3-azabicyclo[3.1.0]hexane-2,4-dione (22). After addition of 30 mg (33.5 mmol) of dibenzoyl peroxide, a mixture of 7.2 g (33.5 mmol) of **21** and 6.35 g (35.06 mmol) of *N*-bromosuccinimide in 130 mL of CCl_4 was boiled for 4.5 h under reflux. The solvent was evaporated, and the residue was distributed between ethyl acetate and water. The product of the dried organic phase was crystallized from ether/petroleum ether and yielded 7.3 g of **22**, mp 94–96 °C, contaminated with the corresponding dibromide **23**. Anal. ($C_{13}H_{12}BrNO_2$) C, H, N; Br: calcd, 27.17; found, 28.4.

1-[4-(Hydroxymethyl)phenyl]-3-azabicyclo[3.1.0]hexane-2,4-dione (24) and 1-(4-Formylphenyl)-3-azabicyclo[3.1.0]hexane-2,4-dione (25). A solution of 5.1 g (18.1 mmol) of crude **22** in 100 mL of THF and 75 mL of water was kept at 70 °C for 16 h. The THF was distilled off, and the solution was extracted with ethyl acetate. The organic phase was washed with water, dried, and evaporated. The residual oil was chromatographed on silica gel with ethyl acetate/hexane, 1:1. The fraction with R_f 0.15 yielded 250 mg of **25**, and the fraction with R_f 0.04 yielded 2.0 g of **24**.

Enzyme Preparation and Assay Procedures. The reagents and conditions for the assays for inhibitory activity against the CSCC and aromatase enzyme systems were those described in ref 8. The aromatase enzyme was obtained from the microsomal fraction of human placenta. Activity was monitored by measuring the ^3H_2O formed during the conversion of 1β,2β-^{3}H-labeled-testosterone or androstenedione to estrogens. Separation of the ^3H_2O from the steroids was achieved by addition of activated charcoal followed by centrifugation. The K_m values for androstenedione and testosterone are 0.038 and 0.13 μM, respectively, so final concentrations in the assay were 0.38 and 1.5 μM, respectively. The source of the CSCC enzyme was the mitochondrial fraction of bovine adrenal cortex. Activity was assayed by measuring the [^{14}C]isocaproic acid released from the substrate, [26-^{14}C]cholesterol. Chromatography on alumina was used to absorb the steroid while the isocaproic acid filtered through the column. A final substrate concentration of 14 μM (K_m = 4.5 μM) was employed.

All assays were run in duplicate at 37 °C, and samples were removed at 5-min intervals up to a total incubation time of 15 min to ensure linearity of product formation. The compounds to be tested were dissolved in ethanol and added to the assay tubes to give 5% of the total volume. Ethanol alone was added to the control tubes. The I_{50} value is the concentration of inhibitor required to reduce the enzyme activity to 50% of the control value at the final substrate concentrations stated above. The apparent K_m for the substrates and the apparent K_i values were determined from Lineweaver–Burk plots, with the method of least-squares analysis being used to obtain a linear fit to the data. The binding spectra and K_s values were determined as described in ref 10.

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Registry No. **2**, 63162-65-2; **3**, 710-96-3; **4**, 11311-28-7; **5**, 93579-60-3; **6a**, 93579-58-9; **6b**, 93579-65-8; **6c**, 93579-67-0; **6d**, 93579-69-2; **6e**, 93579-72-7; **6f**, 93579-70-5; **6g**, 93579-70-5; **6h**, 93579-74-9; **6i**, 93579-78-3; **6j**, 93610-02-7; **6k**, 93579-34-1; **7i**, 113111-29-8; **8**, 113111-30-1; **9a**, 93579-57-8; **9b**, 93579-64-7; **9c**, 93579-66-9; **9d**, 93579-68-1; **9e**, 93579-71-6; **9f**, 93579-75-0; **9g**, 93610-03-8; **9h**, 93579-73-8; **9i**, 93579-77-2; **9j**, 93579-49-8; **9k**, 93579-33-0; **9l**, 93579-48-7; **10a**, 93579-39-6; **10l**, 93579-47-6; **11**, 93579-43-2; **12**, 113111-31-2; **13**, 13312-88-4; **14**, 93579-61-4; **15**,

93579-62-5; **16**, 93579-63-6; **17**, 93579-59-0; **18**, 113111-32-3; **19**, 113111-33-4; **20**, 113111-34-5; **21**, 113111-35-6; **22**, 113111-36-7; **23**, 113111-37-8; **24**, 113111-38-9; **25**, 113111-39-0; H_2NCONH_2, 57-13-6; H_2C=CHCOOEt, 140-88-5; MeI, 74-88-4; EtI, 75-03-6; C_3H_7I, 107-08-4; C_4H_9I, 542-69-8; $H_3C(CH_2)_4I$, 628-17-1; *i*-C_3H_7I, 75-30-9; *i*-C_4H_9I, 513-38-2; $(CH_3)_3CCH_2I$, 15501-33-4; $C_6H_5CH_2Cl$, 100-44-7; $H_2NCH_2C(CH_3)_3$, 5813-64-9; H_2NNH_2, 302-01-2; H_2C=$CHCH_2Br$, 106-95-6; $HOCH_2CH_2NH_2$, 141-43-5; aromatase, 9039-48-9; CSCC enzymes, 37292-81-2; propargyl bromide, 106-96-7.

Coupling Products of Amino Acids to Penicillin V and Cephalothin: Synthesis and Susceptibility to Carboxypeptidases and Lysosomal Enzymes

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Amino acids have been coupled to the carboxyl group of penicillin V and cephalothin by methods that keep the β-lactam ring intact. Derivatives were successfully obtained with both neutral (Leu, Val, Ala, Ile, Trp, Tyr, Gly) and one acidic (Glu) amino acids. The new compounds were inactive in vitro against *Staphylococcus aureus* or *Micrococcus luteus*. Incubation in the presence of purified carboxypeptidases (A, B), soluble lysosomal fractions from liver, or cellular homogenates from liver, kidney, fibroblasts, and macrophages did not allow recovery of the antibacterial activity. Injection in mice also failed to cause liberation of microbiologically active compounds. HPLC studies confirmed that the amide linkage between the antibiotic and the amino acid was not hydrolyzed in the presence of soluble lysosomal fractions from liver. However, conversion of cephalothin and cephalothin-leucine to desacetyl derivatives was observed in the presence of soluble lysosomal fractions and extracts from liver and semipurified orange peel acetylesterase(s). It is concluded that amino acid derivatives of β-lactam antibiotics do not offer potential chemotherapeutic use as prodrugs.

β-Lactam antibiotics are of considerable value in the chemotherapy of bacterial infections because of their great potency, wide spectrum of activity, and low incidence of adverse reactions.[1] These drugs, however, do not accumulate in phagocytic cells[2-5] and, accordingly, are not efficacious against most intracellular bacteria.[6-8] Thus, bacteria that have been ingested by phagocytes but have not been killed by the natural host defense mechanisms appear largely protected from β-lactams. This may result in the development of chronic or recurrent infections.[9,10] β-lactam antibiotics display a free carboxyl group, and this acidic character is probably responsible for their lack of intracellular accumulation, as is observed for other weak organic acids.[11,12] Indeed, conversion of penicillin G into a basic derivative by amidation of its free carboxyl group with a (dimethylamino)propyl moiety results in a considerable enhancement of its accumulation by phagocytic cells and its partial localization in lysosomes.[13] This behavior is consistent with the models proposed to explain the unequal distribution of weak bases and weak acids across membranes separating compartments of different pH.[14] These observations allow the conception of various β-lactam prodrugs, hopefully with enhanced intracellular accumulation.[15] These could result from the coupling of the antibiotic with an amino acid or a small peptide bearing a suitable basic functional group in order to obtain a lysosomotropic drug that would enter cells by diffusion and would subsequently be segregated in lysosomes by proton trapping.[14,15] Coupling could also be made with peptides or proteins that enter cells by endocytosis. These groups would mask the carboxyl group of the β-lactams, while allowing its transport in cells by piggy-back endocytosis.[14-16] Obviously, the validity of these approaches rests upon the capacity for lysosomal enzymes to hydrolyze the amide linkage between the drug and the first amino acid since a free carboxyl group in position 3 of penicillins (or in position 4 of cephalosporins) is essential for antibacterial activity.[17] This paper describes the synthesis of deriva-

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