

Potential Antifertility Agents. 7. Synthesis and Biological Activities of 2-, 3-, and 6-Alkyl-Substituted 4-Aryl-2-methylcyclohexanecarboxylic Acids¹

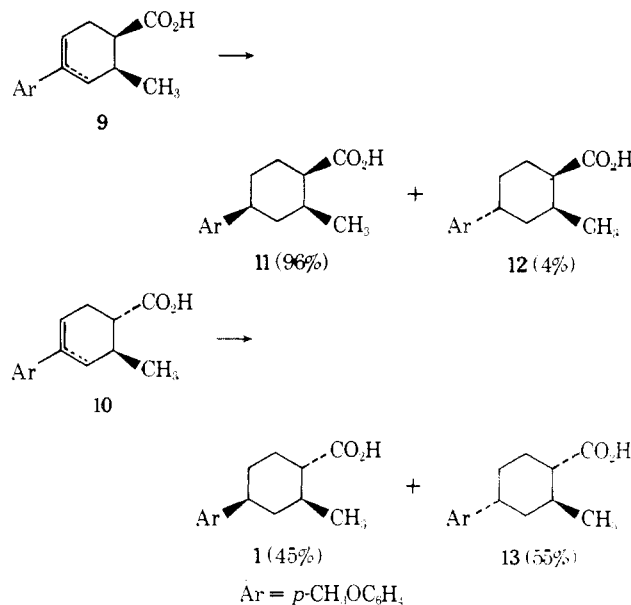
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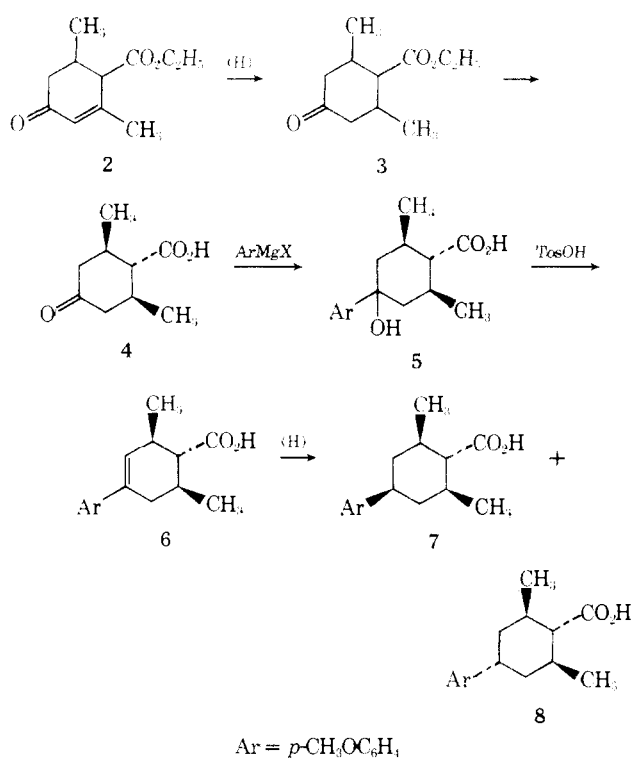
Syntheses and biological activities are reported for 17 of the title compounds. A progressive increase in uterotrophic activity was seen with a systematic placement in proper stereochemical orientations of alkyl groups onto the reference compound, racemic 4β-(*p*-methoxyphenyl)-2β-methylcyclohexane-α-carboxylic acid (1). The activity profile was interpreted in terms of an estrogenic receptor model which had been proposed previously to account for biological activities seen with optical antipodes of 1. The most active compounds of the series were 10,000–18,000 times as active as 1 in uterotrophic assays and prevented pregnancy in mated mice or rats at 0.1 mg/kg postcoitum.

In the previous paper we proposed certain spatial areas on a hypothetical model of an estrogenic receptor site which are involved in the elicitation of a uterotrophic response (Figure 1).¹ This model was invoked primarily to explain the approximately equal uterotrophic potencies seen with the enantiomers of (±)-4β-(*p*-methoxyphenyl)-2β-methylcyclohexane-α-carboxylic acid (1). We now report the synthesis and biological activities of a number of alkylated derivatives of 1. The relative uterotrophic potencies seen with these derivatives lend support to our original hypothesis and additionally give new insights into structural requirements for estrogenic activity.

Chemistry. The first derivative of 1 chosen for evaluation was the methylated derivative 7 which may be viewed as a "hybrid" of (+)-1 and (–)-1. Synthesis of 7 was achieved as outlined in Scheme 1 starting with the keto ester 2² which we found to be a mixture of *cis*-*trans* isomers. Hydrogenation of 2 using a Pd catalyst gave a mixture of isomeric esters 3, from which a single pure keto acid 4 was eventually obtained. This product was converted to the acids 5–8 as indicated. Arguments to support stereochemical assignments of 4–8 are as follows. Dehydration of 5 produced a single (racemic) ene acid 6. This result requires that the methyl groups in 5 (and also in 4



Scheme 1



and 6) be *cis* (since dehydration of 5, which would be expected to occur in two directions, would produce two isomers from a 1,6-*trans*-dimethyl configuration). This assignment is consistent with the result reported by Jacobs, *et al.*, that catalytic reduction with Pt of the keto ester 2 is stereospecific to the extent that both methyl groups in 3 are *cis*.³ Catalytic reduction of 6 produced two isomers 7 and 8 in a ratio of about 3 to 1. This result suggests that the carboxyl in 6 is *trans* relative to the methyls because the opposite configuration for the carboxyl should result in only one saturated isomer upon reduction (*viz.* 1,3,2,3,4,3,6,6)† by analogy with the following previous results.⁴

Additionally, treatment of a mixture of 7 and 8 (as methyl esters) with KO-*t*-Bu in refluxing *t*-BuOH resulted in essentially no change in the isomeric composition; a carboxyl *cis* relative to the *cis* methyls would be expected to epimerize.⁴ Basic hydrolysis of a mixture of 7 and 8 esters resulted in a preferential hydrolysis of the isomer which corresponded to the minor isomer from the catalytic reduction. This result led to our assignment of the stereochemistry of 7 and 8 as shown. Although preferred conformations of both 7 and 8 involve conformers with an equatorial carboxyl, epimer 8 should have a greater population than 7 of a conformer with an axial carboxyl, and it appears to us that hydrolysis should occur easier at an

**Cf.* ref 1 for explanations of the α and β designations. All unsymmetrical structures discussed herein are racemic unless otherwise noted; for brevity only one enantiomeric form is shown in the schemes and tables. To facilitate discussion, 9, 11, and 12 are depicted in the text in different enantiomeric forms than those shown in Table I.

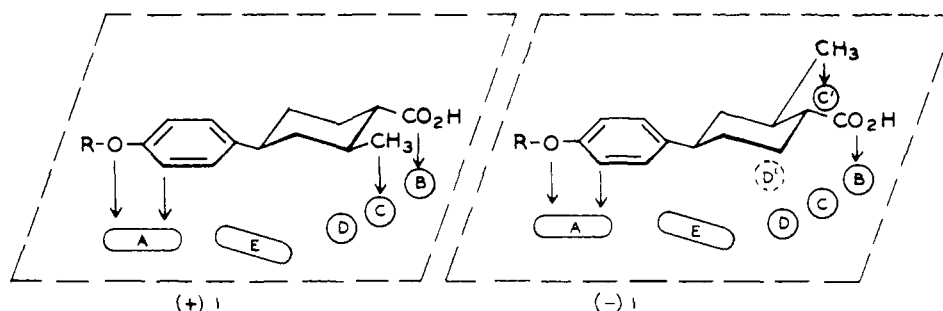
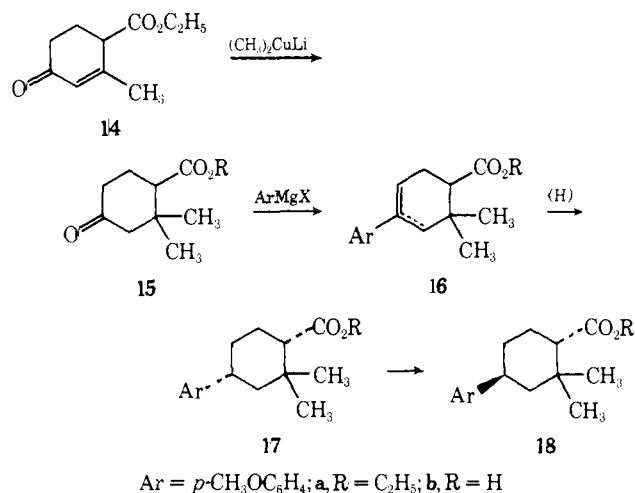


Figure 1. Hypothetical model of estrogenic receptor showing imposition of optical isomers of (\pm)-1 on proposed areas A-E involved in elicitation of biological response; see ref 1 for further explanation.

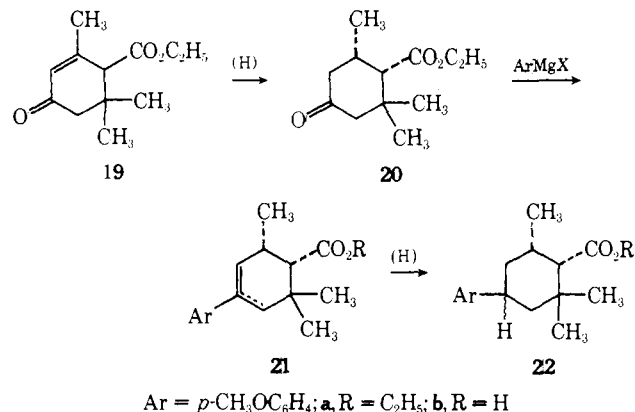
Scheme II



axial carboxyl than an equatorial carboxyl flanked by two equatorial methyl groups.

The biological activity seen with 7 prompted us to extend our studies to the 2,2-dimethyl derivatives 16b and 18b (Scheme II). Earlier work had indicated that either area C or D on the receptor model (Figure 1) could be occupied for estrogenic response,^{1,4} but no indication is available as to whether these areas can be occupied simultaneously. The 2,2-dimethyl acid 18b seemed capable of answering this question since it may be viewed as a "hybrid" of the biologically active diastereomers 1 and 12 (*cf.* 12 as depicted in Table I). The requisite keto acid 15b proved readily accessible starting from Hagemann's ester 14 and lithium dimethylcuprate.^{5,6} This provided a more direct route to 15b than a previously reported synthesis.⁷ Condensation of 15b with the Grignard reagent followed

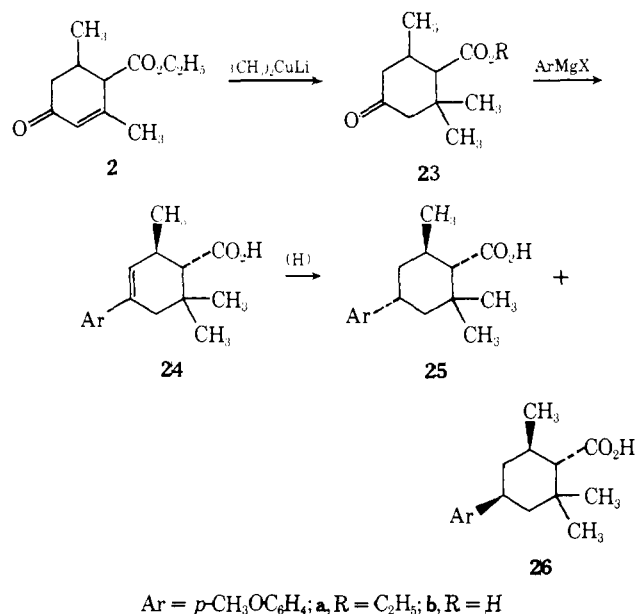
Scheme III



by dehydration of the intermediate hydroxy acid resulted in 16b, a 3:1 mixture of Δ^4, Δ^3 isomers. Hydrogenation of 16 (acid or ester) produced an 80/20 mixture of *cis*-*trans* isomers from which the pure *cis* acid 17b was obtained. Epimerization of the *cis* ester 17a resulted in a 20/80 *cis*-*trans* mixture of esters. Hydrolysis of this mixture gave an acid enriched still further in the *trans* isomer from which the pure *trans* acid 18b was readily obtained. It is noteworthy that the preferential hydrolysis observed is consistent with our isomeric assignments since it is generally accepted that equatorial esters (preferred conformer in 18a) saponify faster than axial esters (probable preferred conformer in 17a).

Schemes III and IV represent a continuation of a systematic placement of methyl groups on the parent structure 1. Catalytic hydrogenation of the ester 19⁸ gave essentially

Scheme IV



one isomer which we have assigned as the *cis* configuration 20. Treatment of the ester 20 with the Grignard reagent resulted in the *cis*- Δ^3, Δ^4 -ene esters 21a. Basic hydrolysis resulted in a low yield of the acid 21b. Catalytic reduction of 21b gave approximately a 2:1 mixture of C-4 epimers (22b). The sample of 22b which was used for biological testing was an 80:20 mixture of C-4 epimers which was ultimately obtained from treatment of the saturated ester 22a (from reduction of 21a) with lithium 1-propanethiolate.⁹

Treatment of the ester 2 (Scheme IV) with lithium dimethylcuprate resulted in a 60/40 mixture of isomeric esters (23a), the minor component of which was identical

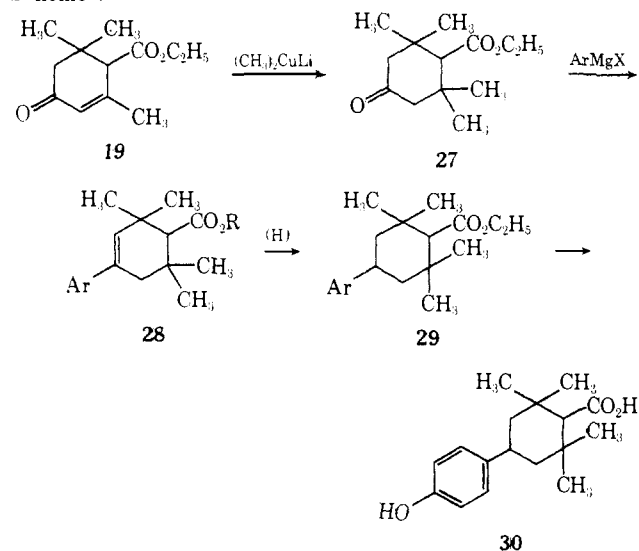
Table I. Relative Uterotropic Potencies of Various 2-, 3-, and 6-Alkyl-Substituted 4-(*p*-Methoxyphenyl)cyclohexene- and -cyclohexanecarboxylic Acids^a

<p>9 (1) 10 (1)</p>	<p>11 (NA) 12 (1)</p>	<p>13 (NA) 14 (1)</p>	<p>R = H^c or CH₃^d (7500-12500)</p>
<p>6 (3-5) 7 (10-20)</p>	<p>8 (NA) 9 (NA)</p>	<p>10 (NA) 11 (NA)</p>	<p>12 (20) 13 (<10)</p>
<p>14 (10-20) 15 (10-20)</p>	<p>16 (10-20) 17 (NA)</p>	<p>18 (NA) 19 (NA)</p>	<p>20 (7500-12500) 21 (7500-12500)</p>
<p>22 (10-20) 23 (10-20)</p>	<p>24 (750-1250) 25 (10-20)^e</p>	<p>26 (NA)^f 27 (10-20)^f</p>	<p>28 (3000-7500) 29 (10,000-18,000)</p>
<p>30 (1500-4000) 31 (1500-4000)</p>	<p>32 (20) 33 (<10)</p>	<p>34 (7500-12500) 35 (7500-12500)</p>	<p>36 (10,000-18,000) 37 (10,000-18,000)</p>
			<p>Ethinylestradiol 2500-3000 Diethylstilbestrol 3000-7500</p>

^a Numbers in parentheses refer to uterotrophic potencies relative to compound 1 in immature 21-day-old rats (*cf.* ref 4 for methodology). All unsymmetrical structures represent racemates. Ar = *p*-CH₃OC₆H₄; NA means not active at 2500 μg unless otherwise noted. ^bA. Mebane, U. S. Patent 3,344,147 (1967). ^cReference 13. ^dAn 80/20 mixture of C-4 epimers; highest dose tested was 250 μg at which no indication of activity was seen; the chemical precursor 21b showed a good response at the 250-μg dose level. ^eThe response seen here may be due to a small contamination with the 4β epimer.

(glpc) with the *cis* ester 20. Hydrolysis of 23a resulted in the keto acid 23b (which was enriched relative to 23a in respect to the *trans* component). This acid was elaborated into a pure Δ³-ene acid which proved different from 21b

Scheme V



Ar = *p*-CH₃OC₆H₄; a, R = C₂H₅; b, R = H

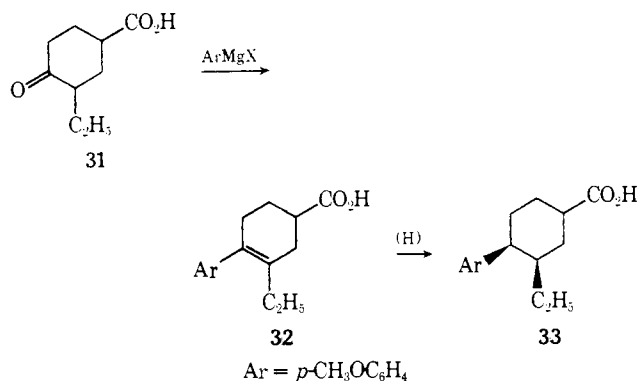
and was thus assigned the *trans* structure 24. Models suggested that catalytic reduction of 24 with hydrogen addition from the less hindered face should result in predominantly the α-aryl isomer 25. Experimentally, it was found that reduction of 24 produced a 90/10 mixture of C-4 epimers; we therefore assigned the major and minor products as the 4α (25) and 4β (26) epimers, respectively. These isomers proved different (glpc analysis of methyl esters) from the two C-4 epimers 22 obtained *via* Scheme III.

The tetramethyl derivative 28a was obtained as indicated in Scheme V. Prolonged basic hydrolysis of 28a gave a low yield of the corresponding acid 28b. Hydrogenation of 28a gave a mixture (2:1) of saturated esters 29. Cleavage of the ester 29 with lithium 1-propanethiolate in HMPA resulted in concomitant cleavage of the methyl ether, a result which was not unexpected.^{10,11} The product 30 which was screened biologically was a mixture of isomers in an approximate ratio of 60:40.

Scheme VI indicates the preparation of the acids 32 and 33 from the known keto acid 31.¹² The saturated acid 33 is a mixture thought to be C-1 epimers.

Schemes VII and VIII depict synthetic routes used to obtain monomethyl and dimethyl analogs of the known potent estrogens 3-ethyl-4-(*p*-methoxyphenyl)-2-methyl-Δ⁴- (and Δ³-) cyclohexenecarboxylic acids.^{13,14} Esters 2 and 19 were alkylated analogously to procedures reported

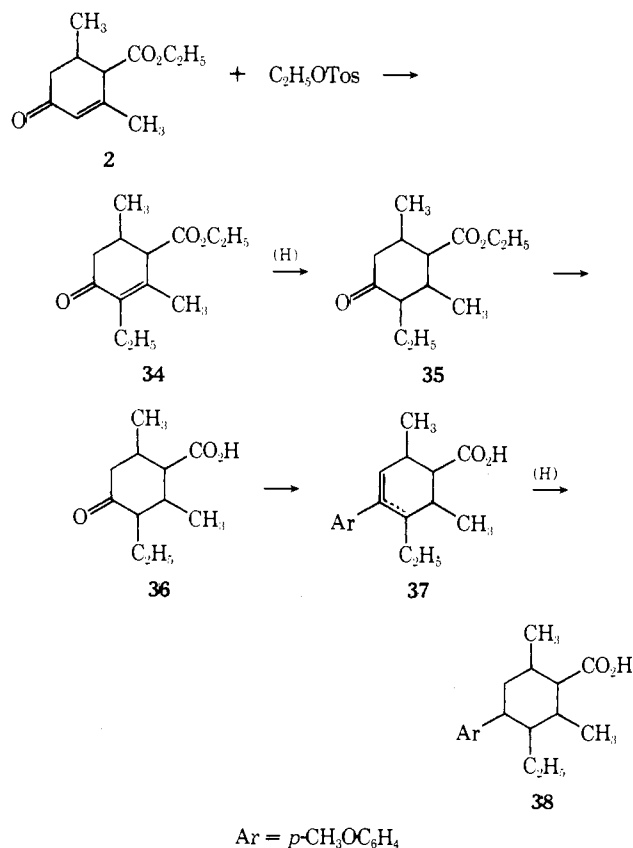
Scheme VI



for alkylation of Hagemann's ester.^{15,16} The acid 37 was a 50/50 mixture of Δ^3 and Δ^4 isomers but was unknown in regard to configurational composition. The saturated relative 38 was similarly unknown in regard to isomeric content. The acid 41 was obtained directly from reaction of the keto ester 40 with the Grignard reagent (after the usual treatment with acid), probably *via* intermediate lactone formation. The yield of 41 thus obtained was very low. The trans structure for 41 was assigned on the basis of nmr analysis which indicated trans-diaxial coupling for protons at C-1 and C-2. Additional work-up of the Grignard reaction mixture resulted in an ester which, after purification, was indicated by nmr and ir analysis to be the Δ^2 ester 42. It is unknown whether isomerization of the double bond occurred during the purification or at an earlier stage. The Δ^2 -ene acid 43 was obtained from 42.

Biology. Methodology for the biological assays has been described previously.⁴ All data are from oral dosing of aqueous solutions of sodium salts of the acids. Estrogenic data are summarized in a format (Table I) which provides

Scheme VII

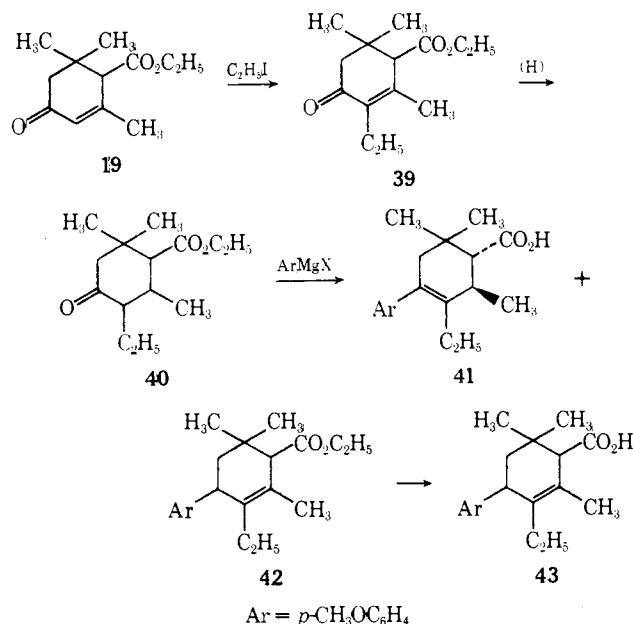


for depiction of trends seen in uterotrophic activity with the various methylated derivatives of 1. For this purpose uterotrophic activities are expressed as potencies relative to 1 which is assigned as unity. Four to six dose levels were examined for each active compound and the derived dose-response curve compared with that of 1 for an estimate of relative potency.

As indicated previously,⁴ cyclohexene acids related to 1 (*cf.* 9 and 10) are of approximately equal uterotrophic potency with 1. The various new cyclohexene derivatives prepared thus provide an additional end point for evaluating the effect of the placement of methyl groups on the parent structure.

The 2,6-dimethyl derivative 7, which may be viewed as a "hybrid" of (+)- and (-)-1, had uterotrophic activity

Scheme VIII



about 10-20 times that of 1. The fact that activity was not only retained but was enhanced lends support to our earlier hypothesis¹ concerning the involvement of a spatial area C' on an estrogenic receptor surface (*cf.* Figure 1) in the elicitation of a uterotrophic response. An enhancement in activity was also seen in the related cyclohexene 6. The lack of activity seen with 8 is expected by analogy with the inactive isomers 11 and 13 which possess a similar cis relationship between the aryl and carboxyl groups. The activity relationship between 7 and 8 complements arguments made above for their stereochemical assignments. A 10- to 20-fold enhancement of activity relative to 1 was seen in the 2,2-dimethyl derivatives 16b and 18b. In terms of our working receptor model (Figure 1), this indicates that areas C and D may not only be occupied simultaneously but that such an arrangement actually results in enhancement of response. Again, total inactivity (2500 μ g) of the C-4 epimeric 17b supports the stereochemical assignments made above.

Acid 24 may be viewed as a combination of structures 6 and 16b, each of which showed enhancement of activity over the parent reference compound 10. As noted in the table, a large synergistic effect is seen with a 750-1250 increase in potency relative to 10. Interestingly, a similarly large increase in potency was not seen with 21b. The activity level seen with acid 25 may be due to a small contamination with its C-4 epimer which we would expect, analogously with 24, to have high activity.

In terms of the receptor model (Figure 1), 24 may be

viewed as occupying simultaneously either areas C, D, and C' or areas C, C', and D'. The latter area D' represents an area complementary to D, capable of accepting axial alkyl groups. Its spatial relationship with D is analogous to the enantiomeric relationship between C and C'.

The vast enhancements in activity seen with the tetramethyl derivatives **28b** and **30** lend credence to the existence of an area D' and suggest that areas C, C', D, and D' may accommodate alkyl groups simultaneously with a resultant increase in biological response. The discrepancy between activities of **21b** and **24** suggests that these areas differ in their relative importance in terms of complexation for maximal response. Biological results with optical isomers of some of the methylated derivatives should shed light on this question.

Compounds **37**, **38**, and **41** represent methylated derivatives of the known potent estrogen 3-ethyl-4-(*p*-methoxyphenyl)-2-methyl- Δ^4 -cyclohexenecarboxylic acid.¹³ It is of considerable interest that **37** and **38**, although mixtures of isomers, maintain the high level of estrogenicity of the parent compound. An exceptionally high level of activity was seen with the derivative **41**. A total dose over 3 days of 0.3 μ g of racemic **41** produced a uterine weight (114.8 mg) greater than that (95.6 mg) obtained with 1.0 μ g of diethylstilbestrol (vehicle control uteri weight 20.8 mg).

Hypocholesterolemic activities of the acids in Table I generally paralleled their trends in estrogenicity. In the normal rat hypocholesterolemic assay,⁴ representative results were **6** (5 mg/kg/day; -51% change in serum cholesterol; $p < 0.05$); **16b** (1; -43%; $p < 0.05$); **24** (0.05; -88%; $p < 0.001$); **28b** (0.05; -72%; $p < 0.05$); **30** (0.05; -75%; $p < 0.05$). Similarly, antifertility activities in various pre- and postcoital dosing regimens generally paralleled the estrogenic trends seen in Table I. Acid **24** was completely effective in preventing pregnancy in mated mice or rats dosed on days 1-5 postcoitum at 0.1 mg/kg/day. Acid **41** was completely effective in the same dosing regimen in rats at 0.1 mg/kg; an occasional pregnancy was seen at 0.05 mg/kg. Acid **41** was not tested for antifertility activity in mice.

In summary, the data in Table I indicate a remarkable increase in uterotrophic activity with the systematic placement in proper stereochemical orientations of alkyl groups on the parent structure **1** and thereby lend support to our proposed estrogenic receptor model.

After this work was completed, Akhrem, *et al.*, reported the synthesis of 4-(*p*-methoxyphenyl)-2,3,6-trimethyl- Δ^4 -cyclohexenecarboxylic acid, but no biological activity was given.¹⁷

Experimental Section†

Melting points (capillary) and boiling points are uncorrected. All compounds had consistent ir and nmr spectra for assigned structures. Routine nmr analyses were obtained using a Varian A-60 spectrometer, while nmr data reported for compounds **41** and **43** were obtained on a Varian HA-100 spectrometer. Mass spectral data were obtained on a LKB 9000 mass spectrometer. Where elemental analyses are indicated by symbols of the elements, analytical results were within $\pm 0.4\%$ of the theoretical values.

2 β ,6 β -Dimethyl-4-ketocyclohexane- α -carboxylic Acid (4). A solution of ethyl 2,6-dimethyl-4-keto- Δ^2 -cyclohexenecarboxylate² (22.7 g, 0.116 mol) in EtOH (300 ml) containing 5% Pd/C (0.8 g) was shaken under an initial hydrogen pressure of 50 psi for 2 hr. Removal of the catalyst and evaporation of the solvent gave a quantitative yield of ethyl 2,6-dimethyl-4-ketocyclohexanecarboxylate (**3**). A solution of **3** (22.2 g, 0.112 mol) in MeOH (60 ml) and H₂O (60 ml) containing KOH (9.35 g, 0.167 mol) was stirred at 25° for 2 hr. Most of the MeOH was removed and the solution was extracted with CH₂Cl₂. The aqueous layer contained 1.96 g (10%) of a mixture of three isomeric acids; this product was discarded. The CH₂Cl₂ was evaporated and the residue was dissolved in EtOH (325 ml) and H₂O (65 ml) containing KOH (8.75 g, 0.156

mol). The solution was heated at reflux for 16 hr and then was worked up as above. The aqueous layer was acidified (3 *N* HCl) and then was extracted with Et₂O. Evaporation of the Et₂O left 13.07 g (69%); recrystallization (MeCN) gave 7.59 g (40%) of **4**, mp 162-164°. *Anal.* (C₉H₁₄O₃) C, H.

4-(*p*-Methoxyphenyl)-2 β ,6 β -dimethyl- Δ^3 -cyclohexene- α -carboxylic Acid (6). A Grignard solution, prepared from *p*-bromoanisole (16.6 g, 0.09 mol) and Mg (2.16 g, 0.09 g-atom) in THF (75 ml), was added to a solution of the keto acid **4** (7.39 g, 0.04 mol) in THF (100 ml) with ice cooling. The mixture was stirred at reflux for 18 hr. Aqueous 1 *N* HCl (100 ml) was added with cooling and then the THF was removed (15 mm). The aqueous residue was extracted with Et₂O. Washing the Et₂O with H₂O caused precipitation of the intermediate hydroxy acid **5** (5.2 g) which was separated by filtration; a 1.5-g aliquot was recrystallized (MeCN) to give **5**, mp 207-208°. *Anal.* (C₁₆H₂₂O₄) C, H. The remainder of **5** plus 9.7 g of semisolid isolated by evaporation of the Et₂O were dissolved in toluene (120 ml) containing *p*-TosOH-H₂O (0.8 g). The solution was refluxed for 2 hr with provision for azeotropic removal of H₂O. Standard work-up⁴ yielded as the crude product an acid (7.37 g, 75%), mp 138-140.5°, which appeared to be homogeneous (glpc analysis after CH₂N₂); recrystallization (MeCN) gave **6**, mp 142-143°. *Anal.* (C₁₆H₂₀O₃) C, H.

4 β -(*p*-Methoxyphenyl)-2 β ,6 β -dimethylcyclohexane- α -carboxylic Acid (7). A solution of **6** (6.00 g, 0.023 mol) in absolute EtOH (300 ml) containing 5% Pd/C (0.6 g) was shaken under an initial H₂ pressure of 50 psi for 16 hr. Removal of the catalyst and evaporation gave a quantitative yield of a mixture of **7** and **8** in a ratio of 3:1 (determined by glpc,† after CH₂N₂ treatment; retention times 6.45 and 7.4 min for **8** and **7**, respectively). A 2.0-g aliquot was recrystallized (MeCN) to give pure **7** (0.5 g), mp 131.5-132.5°. *Anal.* (C₁₆H₂₂O₃) C, H.

4 α -(*p*-Methoxyphenyl)-2 β ,6 β -dimethylcyclohexane- α -carboxylic Acid (8). The crude acids (3.71 g) from the above reduction of **6** were converted to methyl esters by treatment with CH₂N₂. The mixture was refluxed for 19 hr in *t*-BuOH containing KO-*t*-Bu. Usual work-up⁴ gave a product essentially unchanged in isomeric content. A 2.77-g (0.01 mol) quantity of this product in EtOH (40 ml) and H₂O (8 ml) containing KOH (1.30 g, 0.02 mol) was heated at reflux for 20 hr. Usual work-up⁴ gave 0.61 g (23%) of acids **7** and **8** in a ratio of 1:4, respectively. (The unhydrolyzed ester was shown to be pure **7** methyl ester.) Recrystallization (MeCN) gave **8**, mp 161.5-164°, of >95% purity. *Anal.* (C₁₆H₂₂O₃) C, H.

2,2-Dimethyl-4-ketocyclohexanecarboxylic Acid (15b). To a solution of 0° of lithium dimethylcuprate⁹ (0.88 mol) in Et₂O (1 l.) there was added, dropwise with stirring, a solution of Hagemann's ester **14** (73.0 g, 0.40 mol) in Et₂O (500 ml). The mixture was stirred at 22° for 40 min and then was poured into an aqueous solution (pH 8-10) of NH₃ and NH₄Cl. The resultant mixture was extracted several times with Et₂O, and the combined extracts were washed with water and dried (Na₂SO₄). Removal of the solvent and distillation of the residue gave 68.1 g (81%), bp 72-85° (0.1-0.2 mm), of ethyl 2,2-dimethyl-4-ketocyclohexanecarboxylate (**15a**) containing about 10% of unchanged Hagemann's ester. The latter was removed in the next step since it is unstable to basic hydrolysis.¹⁵

A solution of the product (68.0 g, 0.34 mol) in EtOH (400 ml) containing KOH (23.0 g, 0.4 mol) and water (80 ml) was heated at reflux for 5 hr. The alcohol was removed at 10 mm and the solution was extracted with Et₂O. The aqueous phase was cooled and acidified (3 *N* HCl). The title acid was extracted into Et₂O and the solution was washed, dried, and evaporated to leave 46.6 g; distillation gave **15b** (33.2 g, 57%) of bp 113-119° (0.2 mm) as a waxy, hygroscopic solid [lit.⁷ bp 160-170° "bath temperature" (0.4 mm)].

4-(*p*-Methoxyphenyl)-2,2-dimethyl- Δ^4 (Δ^3)-cyclohexenecarboxylic acid (16b) was prepared from the acid **15b** and 2 equiv of *p*-methoxyphenylmagnesium bromide as described above for **6**. The yield of **16b** after recrystallization (MeCN) was 53%; mp 137.5-139° (previous softening from 135°); shown by nmr analysis of the vinylic proton pattern to be a mixture of Δ^4 : Δ^3 in approximate ratio of 3:1, respectively. *Anal.* (C₁₆H₂₀O₃) C, H.

***cis*-4-(*p*-Methoxyphenyl)-2,2-dimethylcyclohexanecarboxylic Acid (17b).** Catalytic reduction of **16b** (4.00 g) in EtOH (100 ml) containing 5% Pd/C (0.5 g) gave quantitatively an 80:20 mixture of **17b** and the trans isomer **18b**, respectively (glpc† retention

† Glass column, 6 ft \times 6 mm i.d., packed with 3% butane-1,4-diol succinate polyester on 100-120 Gas-Chrom Q (Applied Science Laboratories, Inc.) at 190° isothermally, flow rate of 60 ml of He/min.

times, Me esters, 7.0 and 9.25 min, respectively). Recrystallization (MeCN) gave 2.83 g containing about 12% **18b**. This product was dissolved in hot EtOH (25 ml); as the solution cooled, **18b** crystallized as beads. The solution was observed further until formation of white needles was seen; at this point the supernatant was decanted and allowed to stand at 25° for 16 hr. Filtration gave pure **17b** (1.23 g, 31%), mp 153–154.5°. *Anal.* (C₁₆H₂₂O₃) C, H.

trans-4-(*p*-Methoxyphenyl)-2,2-dimethylcyclohexanecarboxylic Acid (**18b**). The ethyl ester **16a** (prepared by Fischer esterification of **16b**) was hydrogenated as above to an 80:20 mixture of *cis*-*trans* isomers. A solution of the product (7.50 g, 0.026 mol) in *t*-BuOH (75 ml) containing KO-*t*-Bu (from 0.34 g, 0.009 g-atom) was refluxed for 17 hr. Usual work-up gave a product (5.93 g, 81%) indicated by glpc to be a 20:80 *cis*-*trans* mixture. Hydrolysis in refluxing aqueous ethanolic KOH for 16 hr as described above gave as the crude product 3.21 g (59%) of an acid containing 95% of the *trans* isomer **18b**. Recrystallization (EtOH) gave pure **18b**, mp 171–173°. *Anal.* (C₁₆H₂₂O₃) C, H.

Ethyl *cis*-4-keto-2,2,6-trimethylcyclohexanecarboxylate (**20**) was prepared from reduction of ethyl 2,6,6-trimethyl-4-keto-2-cyclohexene-1-carboxylate⁸ (**19**) as described above for **4**. The crude product (100%) was used directly in the next step; glpc \ddot{s} (retention time, 10.35 min) indicated 99% purity.

cis-4-(*p*-Methoxyphenyl)-2,6,6-trimethyl- $\Delta^3(\Delta^4)$ -cyclohexanecarboxylic Acid (**21b**). The ethyl ester **21a** was prepared from the keto ester **20** and 1 equiv of *p*-methoxyphenylmagnesium bromide as described above for **6**. The yield of **21a**, bp 141–143° (0.05 mm), was 43%. *Anal.* (C₁₉H₂₆O₃) C, H. A solution of **21a** (9.69 g, 0.032 mol) in EtOH (100 ml) and H₂O (20 ml) containing KOH (9.00 g, 0.16 mol) was refluxed for 5 days. Standard work-up gave 2.40 g of crude acid which was recrystallized twice from MeCN to give **21b** (0.86 g, 10%), mp 134–136° (softening at 132°) shown by glpc (after CH₂N₂ treatment) to be a 95:5 mixture of *ene* isomers; the nmr spectrum showed a multiplet pattern for the vinylic proton which appeared consistent for the Δ^3 isomer as the major component. *Anal.* (C₁₇H₂₂O₃) C, H.

4-(*p*-Methoxyphenyl)-2,2,6- α -trimethylcyclohexane- α -carboxylic Acid (**22b**). Catalytic hydrogenation of the ester **21a** (12.40 g, 0.041 mol) in EtOH (200 ml) containing 5% Pd/C (1.20 g) gave quantitatively a 90/10 mixture of the C-4 epimeric esters **22a**. A solution of **22a** (6.67 g, 0.022 mol) and 150 ml of 0.45 M lithium 1-propanethiolate in HMPA⁹ was stirred under nitrogen at 25° for 40 hr. The mixture was poured onto 0.5 N NaOH and was extracted several times with Et₂O. The aqueous phase was acidified and then extracted with fresh Et₂O. Work-up gave 2.00 g of acid; two recrystallizations (MeCN) gave 0.64 g (11%) of **22b**, mp 139.5–141.5° (softening from 135°), indicated by glpc (after CH₂N₂) to be an 80:20 mixture. *Anal.* (C₁₇H₂₄O₃) C, H.

4-Keto-2,2,6-trimethylcyclohexanecarboxylic Acid (**23b**). The ester **23a** was prepared from the ester **2²** and lithium dimethylcuprate⁵ by the procedure described above for **15a**; yield, 67%; bp 83–90° (0.3–0.5 mm); indicated by glpc \ddot{s} to be a 38:62 mixture of *cis*-*trans* isomers (retention times, 10.35 and 11.10 min, respectively). A solution of **23a** (24.00 g, 0.113 mol) in ethylene glycol (57 ml) and H₂O (7 ml) containing KOH (6.93 g, 0.136 mol) was refluxed for 18 hr. Usual work-up gave a mixture (15.68 g, 76%) of isomeric acids (**23b**), approximate 1:3 *cis*-*trans* content; an analytical sample was prepared by short-path distillation, bp 142–148° (0.2 mm). *Anal.* (C₁₀H₁₆O₃) C, H.

trans-4-(*p*-Methoxyphenyl)-2,6,6-trimethyl- Δ^3 -cyclohexanecarboxylic acid (**24**) was prepared from the keto acid **23b** and 2 equiv of *p*-methoxyphenylmagnesium bromide as described above for **6**. Two recrystallizations (MeCN) of the crude product gave **24** (yield, 21%); mp 165–166.5°; shown to be different (glpc after CH₂N₂) from either of the isomers of **21b**; as with **21b**, the nmr spectrum suggested the Δ^3 assignment. *Anal.* (C₁₇H₂₂O₃) C, H.

4 α -(*p*-Methoxyphenyl)-2,2,6 β -trimethylcyclohexane- α -carboxylic Acid (**25**). The acid **24** (4.00 g, 0.0146 mol) was hydrogenated in EtOH containing 5% Pd/C as previously described to give quantitatively a 90:10 mixture of C-4 epimers assigned as the 4 α and 4 β epimers, respectively. Recrystallization (MeCN–EtOH) gave **25** (98% pure), mp 163.5–167°. *Anal.* (C₁₇H₂₄O₃) C, H.

A sample of the acid **21b** was similarly hydrogenated to yield a 2:1 mixture of C-4 epimers; the mixture was shown by glpc (after CH₂N₂) to be different from the mixture of C-4 epimers obtained from hydrogenation of **24**.

Ethyl 4-keto-2,2,6,6-tetramethylcyclohexanecarboxylate (**27**)

was prepared from the ester **19⁸** and lithium dimethylcuprate⁵ by the procedure described above for **15a**; yield, 82%; bp 86–87° (0.2 mm). *Anal.* (C₁₃H₂₂O₃) C, H.

4-(*p*-Methoxyphenyl)-2,2,6,6-tetramethyl- Δ^3 -cyclohexanecarboxylic Acid (**28b**). The ethyl ester **28a** was prepared from the keto ester **27** and 1 equiv of *p*-methoxyphenylmagnesium bromide as described above for **6**; yield of **28a** was 65%; bp 151–154° (0.3 mm). *Anal.* (C₂₀H₂₈O₃) H; C; calcd, 75.91; found, 75.41. A solution of **28a** (12.01 g, 0.038 mol) in EtOH (100 ml) and H₂O (20 ml) containing KOH (11.20 g, 0.20 mol) was heated at reflux for 64 hr. Usual work-up yielded 0.45 g (4%) of acid **28b**, mp 142–144° after recrystallization (MeCN). *Anal.* (C₁₈H₂₃O₃) C, H.

Ethyl 4-(*p*-Methoxyphenyl)-2,2,6,6-tetramethylcyclohexanecarboxylate (**29**). Catalytic reduction of the ester **28a** in EtOH containing 5% Pd/C gave quantitatively **29** as a mixture (*ca.* 2:1) of *cis*-*trans* isomers; bp 152–155° (0.3 mm). *Anal.* (C₂₀H₃₀O₃) C, H.

4-(*p*-Hydroxyphenyl)-2,2,6,6-tetramethylcyclohexanecarboxylic Acid (**30**). Treatment of the ester **29** with lithium 1-propanethiolate at 25° as described above for **22a** resulted in insignificant amounts of cleavage. The quality of the reagent was shown to be satisfactory when we repeated the reported⁹ quantitative cleavage of methyl mesitoate. The reagent then was condensed with **29** in HMPA at 65° for 21 hr. Work-up as described above gave **30**; yield, 38%, after trituration under CH₂Cl₂; mp 174–178° (*ca.* 60:40 mixture of *cis*-*trans* isomers). *Anal.* (C₁₇H₂₄O₃) C, H.

3-Ethyl-4-(*p*-methoxyphenyl)- Δ^3 -cyclohexanecarboxylic acid (**32**) was prepared from 3-ethyl-4-ketocyclohexanecarboxylic acid¹² by the procedure described above for **6**; yield of **32**, 43%; mp 127–129° (after CH₂N₂, glpc indicated 98% pure). *Anal.* (C₁₆H₂₀O₃) C, H.

3-Ethyl-4-(*p*-methoxyphenyl)cyclohexanecarboxylic Acid (**33**). The acid **32** was reduced catalytically in EtOH by the usual procedure described above; yield of **33**, 38% after two recrystallizations from methylcyclohexane; mp 74–80°; shown by glpc (after CH₂N₂) to be a 92:8 mixture of isomers. *Anal.* (C₁₆H₂₂O₃) C, H.

2,6-Dimethyl-3-ethyl-4-ketocyclohexanecarboxylic Acid (**36**). The keto ester **2²** (21.45 g, 0.109 mol) was added over 15 min to a stirred solution of NaOEt (from 2.64 g of Na, 0.115 g-atom) in absolute EtOH (90 ml). The solution was stirred at 25° for 40 min, and then ethyl *p*-toluenesulfonate (23.00 g, 0.115 mol) was added. The mixture was heated under reflux for 3.5 hr. Work-up gave a product (22.36 g) indicated (glpc) to be a mixture of the alkylated ester **34** (56%), unchanged **2** (35%), and unknown impurities (possibly C-1 alkylated products).¹⁶ Pure **34** could not be obtained from distillation (2-ft spinning-band column). The best fractions [bp 77° (0.07 mm)] contained 82% **34** (two diastereomers) and 14% **2** (two diastereomers). Hydrogenation (5% Pd/C in EtOH as above) gave a product which was distilled (2-ft spinning-band column) to give the ester **35** [bp 64–65.5° (0.06 mm), 10.60 g] as a mixture of isomers. Refluxing for 16 hr of **35** in aqueous ethanolic KOH as above yielded **36** (7.59 g, 82%) as an oil (mixture of isomers) which was used in the following preparation.

2,6-Dimethyl-3-ethyl-4-(*p*-methoxyphenyl)- $\Delta^3(\Delta^4)$ -cyclohexanecarboxylic acid (**37**) was prepared from the keto acid **36** as described above for **6**; yield, 2.23 g (20%); mp 144–158°; indicated by nmr to be *ca.* 50/50 mixture of Δ^3 and Δ^4 isomers. *Anal.* (C₁₈H₂₄O₃) C, H.

2,6-Dimethyl-3-ethyl-4-(*p*-methoxyphenyl)cyclohexanecarboxylic acid (**38**) was prepared from catalytic hydrogenation (5% Pd/C in EtOH) of **37**; yield of **38**, 52%, after two recrystallizations (MeCN) to mp 135–155°; glpc (after CH₂N₂) indicated a minimum of four isomers, the major of which comprised about 70% of the total. *Anal.* (C₁₈H₂₆O₃) C, H.

Ethyl 3-Ethyl-4-keto-2,6,6-trimethyl- Δ^2 -cyclohexanecarboxylate (**39**). The ester **19⁸** (25.0 g, 0.119 mol) was added to a solution of KO-*t*-Bu (from 4.64 g of K, 0.119 g-atom) in *t*-BuOH (95 ml) while cooling to maintain the temperature below 38°. A solution of ethyl iodide (18.6 g, 0.119 mol) in *t*-BuOH (20 ml) was added and the mixture was stirred under reflux for 3.5 hr. Usual work-up gave **39** (26.24 g, 93%), bp 86–89° (0.1 mm). *Anal.* (C₁₄H₂₂O₃) C, H.

Ethyl 3-Ethyl-4-keto-2,6,6-trimethylcyclohexanecarboxylate (**40**). A solution of **39** (20.1 g, 0.085 mol) in EtOH (90 ml) containing 3 N aqueous HCl (12 ml) and 5% Pd/C (1.1 g) was shaken under H₂ (50 psi initially) until theoretical uptake was observed. Usual work-up gave **40**, bp 64–65° (0.1 mm). *Anal.* (C₁₄H₂₄O₃) C, H.

trans-3-Ethyl-4-(*p*-methoxyphenyl)-2,6,6-trimethyl- Δ^3 -cyclohexanecarboxylic acid (**41**) was prepared from the keto ester **40** and 1 equiv of *p*-methoxyphenylmagnesium bromide as described

[§]Glass column, 6 ft \times 6 mm i.d., packed with 3% OV-17 on 100–120 Gas-Chrom Q (Applied Science Laboratories, Inc.), programmed from 100° at 4°/min, flow rate of 60 ml of He/min.

above for 6. After treatment with *p*-TosOH·H₂O and usual work-up the crude yield of 41 was 5% which recrystallized (MeCN) to pure 41: mp 164.5–167.5°; ir (KBr) 1700 cm⁻¹ (C=O); nmr (CDCl₃) δ 7.0–7.7 (q, 4, *J* = 8.5 Hz, aromatic), 3.78 (s, 3, CH₃O), 2.7 (m, broad, 1, CH₃CH), 2.4–1.8 (overlapping m, 5, CHCO₂H and CH₂C=CCH₂), 1.2–1.05 [doublet overlapping two singlets, *J* = 6 Hz, 9, CHCH₃ and C(CH₃)₂], and 0.87 (t, 3, *J* = 7.5 Hz, CH₂CH₃); scale expansions and spin decouplings confirmed the assignments as well as establishing the stereochemistry; coupling constant of the C-1 proton (10 Hz) was indicative of trans-diaxial coupling; mass spectrum (70 eV) *m/e* 302. *Anal.* (C₁₉H₂₆O₃) C, H.

3-Ethyl-4-(*p*-methoxyphenyl)-2,6,6-trimethyl-Δ²-cyclohexenecarboxylic Acid (43). The Et₂O extracts which remained after the base extractions in the above experiment were worked up to leave an oil. Distillation [bp 173–182° (0.05 mm)] gave the Δ²-ene ester 42 (yield, 42%): nmr (CDCl₃) δ 1.70 (d, 3, *J* = 2 Hz, CH₃C=C). *Anal.* (C₂₁H₃₀O₃) C, H. A solution of the ester 42 (1.10 g, 0.003 mol) in ethylene glycol (18 ml) and H₂O (3.6 ml) containing KOH (2.00 g, 0.036 mol) was heated at reflux for 88 hr. Usual work-up gave the acid 43 (0.67 g, 67%): mp 131–132° (MeCN); ir (KBr) 1697 cm⁻¹ (C=O); nmr (CDCl₃) δ 1.76 (d, 3, *J* = 2 Hz, CH₃C=C); mass spectrum (70 eV) *m/e* 302. *Anal.* (C₁₉H₂₆O₃) C, H.

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Synthesis and Antifolate Activity of Isoaminopterin†

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The synthesis of isoaminopterin has been carried out by two methods. The first parallels the method employed for constructing the isofolate framework recently developed in this laboratory, which involves a novel ring closure of a substituted pyrimidine. The second involves the displacement of the halogen of 2,4-diamino-6-chloropteridine by α-amino-*p*-toluic acid. Isoaminopterin has been tested for its ability to inhibit the growth of two folate-requiring organisms. Inhibition studies of this compound were carried out on the enzyme dihydrofolate reductase. While isoaminopterin is equally potent in its bacteriostatic activity as aminopterin, it is somewhat less inhibitory for dihydrofolate reductase.

As part of a continuing program aimed at the design, synthesis, and biological evaluation of folate analogs which would be less toxic and more specific in their action than currently available drugs, we have recently reported 9-oxofolic acid¹ and isofolic acid.² Although the altered isomeric framework of isofolic acid could contribute specific interference with the metabolic pathways in which 5,10-methylenetetrahydrofolate takes part as a cofactor, its 4-amino analog might also elicit antimetabolic activity by inhibiting the enzyme dihydrofolate reductase.³⁻⁵ Since no 4-amino analogs of isofolic acid have been prepared, the preceding assumption of inhibition of DHFR by

4-aminoisofolate is only speculative and warranted examination. It appeared that although 4-aminoisofolate possesses the 2,4-diamino functions at the pteridine moiety as with the classical aminofols, the reversal of substituents at the C₉-N₁₀ positions could alter its binding characteristics for DHFR, which should be reflected in altered inhibitory characteristics. Some evidence to support this type of reasoning is available. For example, it has been noted that dihydroisofolic acid, which differs from dihydrofolic acid only in the exchange of the amino and methylene groups at positions 9 and 10, does not behave as a substrate for DHFR.‡

Another reason for the synthesis of 4-aminoisofolic acid comes from the work of Kisliuk and coworkers who investigated the ability of the classical aminofols aminopterin,⁶ methotrexate,⁷ and their dihydro and tetrahydro derivatives to inhibit the enzyme thymidylate synthetase and concluded that the tetrahydro forms are more powerful inhibitors than the parent compounds. Since formaldehyde

†Trivial names in general usage will be used for these compounds: isoaminopterin = *N*-[*p*-[[[(2,4-diamino-4-deoxy-6-pteridinyl)amino]methyl]benzoyl]glutamic acid; methotrexate = *N*-[*p*-[[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]glutamic acid; aminopterin = *N*-[*p*-[[[(2,4-diamino-6-pteridinyl)methyl]amino]benzoyl]glutamic acid; folic acid = *N*-[*p*-[[[(2-amino-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl]glutamic acid; homofolic acid = *N*-[*p*-[[[(2-amino-4-hydroxy-6-pteridinyl)ethyl]amino]benzoyl]glutamic acid. Other abbreviations include DHFR, dihydrofolate reductase; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran; DEAE, diethylaminoethyl; DMF, dimethylformamide; Boc, *tert*-butyloxycarbonyl.

‡C. M. Baugh, R. L. Kisliuk, and M. G. Nair, unpublished work, University of South Alabama and Tufts University, 1974.