410 nm/500 nm for DA) and compared with standard curves prepared daily for each catecholamine. Sensitivity was 0.5 ng for NE and 4 ng for EPI, values that were approximately twice that of the blank.

Acknowledgment. We are greatly indebted to D. Conyne, T. E. Milgate, and S. A. McCreedy for their excellent technical assistance, Drs. R. C. Griffith and R. J. Murray for their suggestions during the preparation of this manuscript, and C. Schlosser and E. L. Gooch for the excellent secretarial assistance. We also thank Molecular Design Ltd. for providing us a prereleased version of ChemText to prepare this manuscript.

Registry No. **3**, 79619-31-1; **3**·$C_4H_4O_4$, 79619-32-2; **4**, 84858-20-8; **4**·HCl, 76323-05-2; **5**, 117096-72-7; **5**·HCl, 76323-03-0; **6**, 117096-73-8; **6**·HCl, 117096-95-4; **7**, 117096-74-9; **7**·HCl, 117096-96-5; **8**, 117096-75-0; **8**·HCl, 84858-43-5; **9**, 87272-34-2; **9**·HCl, 84858-30-0; **10**, 84858-33-3; **10**·HCl, 84858-32-2; **11**, 84858-58-2; **11**·HCl, 84858-31-1; **12**, 84858-59-3; **12**·HCl, 84858-39-9; **13**, 84858-35-5; **13**·HCl, 84858-34-4; **14**, 117096-76-1; **14**·HCl, 76323-06-3; **15**, 84858-37-7; **15**·HCl, 84858-36-6; **16**, 117096-77-2; **16**·HCl, 117096-97-6; **17**, 117096-78-3; **17**·HCl, 117096-98-7; **18**, 117096-79-4; **18**·HCl, 117096-99-8; **19**, 117096-80-7; **19**·HCl, 117097-00-4; **20**, 117096-81-8; **20**·HCl, 117097-01-5; **21**, 117096-82-9; **21**·HCl, 117097-02-6; **22**, 117096-83-0; **23**, 117096-65-8; **23**·HCl, 117097-03-7; **24**, 117096-64-7; **25**, 117096-84-1; **25**·HCl, 84858-27-5; **26**, 117096-85-2; **26**·HCl, 84858-25-3; **27**, 117096-86-3; **27**·HCl, 117097-04-8; **28**, 117096-87-4; **29**, 117096-88-5; **29**·2HCl, 117097-05-9; **30**, 117096-89-6; **30**·HCl, 117097-06-0; **31**, 117096-90-9; **31**·HCl, 106287-81-4; **32**, 37933-65-6; **32**·HCl, 38186-01-5; **33**, 117096-91-0; **33**·$C_4H_4O_4$, 117097-07-1; **34**, 117096-92-1; **34**·HCl, 84858-41-3; **35**, 117096-93-2; **35**·HCl, 117097-08-2; **36**, 117096-70-5; **36**·HCl, 117096-71-6; **37**, 117096-68-1; **37**·HCl, 117097-09-3; **38**, 81716-13-4; **38**·HCl, 117097-10-6; **39**, 6665-86-7; **40**, 84858-19-5; **41**, 117096-94-3; **42**, 525-66-6; **43**, 89-84-9; **45**, 66832-97-1; **46**, 5465-06-5; **47**, 117096-61-4; **49** (R′ = H), 117096-63-6; **51**, 117096-66-9; **53a**, 93876-03-0; **53b**, 117096-69-2; **54**, 117096-67-0; **56**, 117096-62-5; 2,3-$(CH_3O)_2C_6H_3COCH_3$, 38480-94-3; 2,3-$(OH)_2C_6H_3COCH_3$, 13494-10-5; epichlorohydrin, 106-89-8; norepinephrine, 51-41-2.

Phenolic Metabolites of Clomiphene: [(*E*,*Z*)-2-[4-(1,2-Diphenyl-2-chlorovinyl)phenoxy]ethyl]diethylamine. Preparation, Electrophilicity, and Effects in MCF 7 Breast Cancer Cells

Peter C. Ruenitz,*[†] Richard F. Arrendale,[‡] Walter F. Schmidt,[†] Carolyn B. Thompson,[†] and Nitin T. Nanavati[†]

College of Pharmacy, University of Georgia, Athens, Georgia 30602, and Tobacco Safety Unit, Agricultural Research Service, United States Department of Agriculture, Athens, Georgia 30613. *Received April 29, 1988*

The triarylethylene antiestrogen clomiphene (**1**) was previously shown to undergo biotransformation to an active metabolite, 4-hydroxyclomiphene (**2**), and to 3-methoxy-4-hydroxyclomiphene (**3**) plus the respective regioisomers of these, **4** and **5**. We now report the synthesis and further chemical and biochemical studies on **3**–**5**. Coupling of 4-[2-(diethylamino)ethoxy]benzophenone (**10**) with either 4-(benzyloxy)benzaldehyde (**11a**) or its 3-methoxy analogue **11b** in the presence of titanium, followed by chlorination and deprotection of the intermediate triarylethylenes, gave **4** and **5**, respectively. Condensation of benzylmagnesium chloride with the (2-methoxyethoxy)methyl (MEM) ether of 4-[2-(diethylamino)ethoxy]-3′-methoxy-4′-hydroxybenzophenone (**8**), followed by mild acid treatment, afforded deschloro **3** due to facile MEM ether hydrolysis. Acetylation of this, followed by chlorination and deacetylation, gave **3**. Compounds **4** and **5** reacted readily with nucleophiles. In particular, 2-mercaptoethanol reacted with **4** to afford deschloro vinyl thioether **13** as suggested by NMR spectral studies, a result that implicated allene–quinone **14** as the electrophilic species produced in solution from **4**. Antiestrogen binding sites and estrogen receptors from MCF 7 human breast cancer cells interacted with **3** and **5** with affinities comparable to those of tamoxifen and **1**, respectively; **5** was shown not to bind irreversibly with these sites. Inhibition of MCF 7 cell proliferation by **3**–**5** at 5 μM concentrations (76%, 57%, and 49%, respectively, relative to drug-free controls) compared favorably to that observed with 5 μM **1** (80%). These results suggest that **3**–**5** as well as **2** may contribute to the antiestrogenic effects of **1**.

Triarylethylene antiestrogens have been used successfully in breast cancer treatment.[1] In experimental studies the antiestrogen clomiphene (**1**)[2] has been shown to suppress the proliferation of cultured human breast cancer cells and to inhibit the growth of chemically induced breast cancer in the rat.[3] Also, clomiphene has been suggested to antagonize estrogen-mediated feedback inhibition of gonadotropin-releasing hormone secretion in the hypothalamus, a mechanism that may account for its ability to induce ovulation.[4]

Interaction with drug-metabolizing enzymes may be a complicating factor in elucidating the molecular mechanism(s) of action of **1**. In the presence of rat liver microsomes **1** underwent N-oxidation, N-deethylation, and conversion to **2**.[5a] The first two metabolites had estrogen receptor (ER) affinities similar to that of **1**, and **2** had much higher ER affinity and greater antiestrogenic and antiproliferative potencies than did **1**.[3,5] Further studies of the metabolism of **1** in the immature female rat have

[†] College of Pharmacy.
[‡] Tobacco Safety Unit.

(1) Lippman, M. E. In *Williams Textbook of Endocrinology*, 7th ed.; Wilson, J. D., Foster, D. W., Eds.; W. B. Saunders: Philadelphia, 1985; pp 1309–1319.
(2) For convenience, the trans configurations of **1** and other triarylethylenes are illustrated.
(3) (a) Murphy, L. C.; Sutherland, R. L. *J. Clin. Endocrinol. Metab.* **1983**, *57*, 373–379. (b) Hubinont, P. O., Leroy, F., Galand, P., Eds. *Basic Action of Sex Steroids on Target Organs*; S. Karger AG: Basel, 1971; pp 274–279.
(4) (a) Huppert, L. C. *Fertil. Steril.* **1979**, *31*, 1–8. (b) Adashi, E. Y. *Fertil. Steril.* **1984**, *42*, 331–344.
(5) (a) Ruenitz, P. C.; Bagley, J. R.; Mokler, C. M. *Biochem. Pharmacol.* **1983**, *32*, 2941–2947. (b) Ruenitz, P. C.; Bagley, J. R.; Watts, C. K. W.; Hall, R. E.; Sutherland, R. L. *J. Med. Chem.* **1986**, *29*, 2511–2519.

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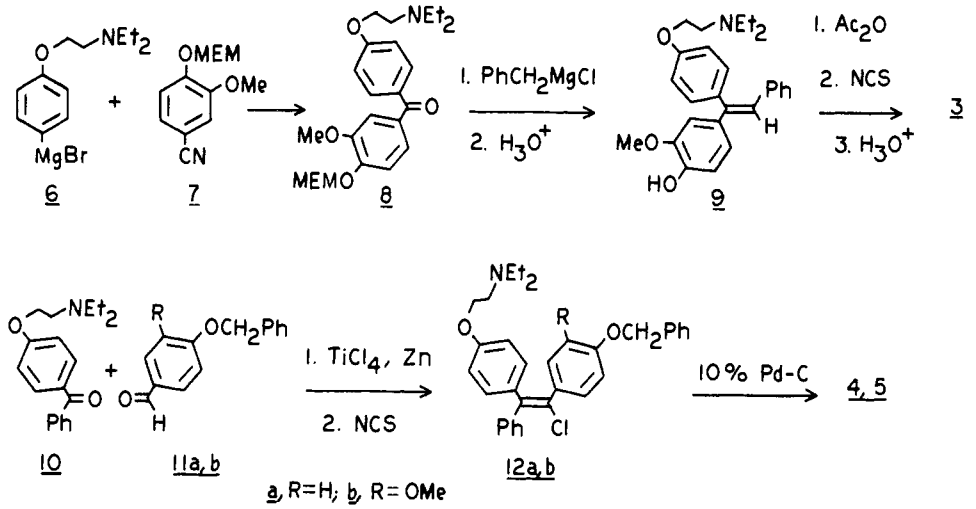
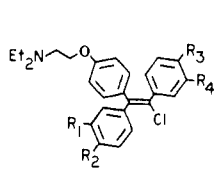
Scheme I



led to the characterization of **3–5** as additional metabolites.[6]



	R_1	R_2	R_3	R_4
1	H	H	H	H
2	H	OH	H	H
3	OMe	OH	H	H
4	H	H	OH	H
5	H	H	OH	OMe

The latent electrophilic properties of two of these metabolites (**4** and **5**), thought to be converted spontaneously, by loss of chloride, to allene–quinones,[6] have prompted us to compare their rates of reaction with selected nucleophiles and to characterize their interactions with ER and with antiestrogen binding sites. Also, we have determined the antiproliferative potencies of **3–5** in order to assess whether these hydroxylated metabolites could contribute to the tumor growth suppressing effect of **1**.

Results

Synthesis of 3–5. Reaction of aryl Grignard reagent **6** with nitrile **7** gave benzophenone **8** in 20% yield (Scheme I). This was allowed to react with benzylmagnesium chloride, followed by dehydration of the resulting carbinol with aqueous HCl, a condition under which (2-methoxyethoxy)methoxy (MEM) ethers of **2** and related compounds were stable,[5b,7] but which in this case resulted in loss of the MEM group, giving a 52% yield of hydroxytriarylethylene **9**. Chlorination of **9** acetate ester with *N*-chlorosuccinimide (NCS) followed by deacetylation with HCl in aqueous methanol gave **3** (44%). Attempted preparation of **12a,b** by reaction of **10**, with suitably substituted benzylmagnesium bromides was not successful. However, coupling of **10** with **11a** or **11b** in the presence of low-valent titanium, a reagent previously shown to afford triarylethylenes via reaction of substituted benzophenones with benzaldehyde or propiophenone,[8] afforded, after treatment of each intermediate with NCS, **12a,b** in overall yields of 32% and 27%, respectively. Hydrogenolysis of each benzyl ether gave **4** and **5** in respective yields of 57% and 68%.

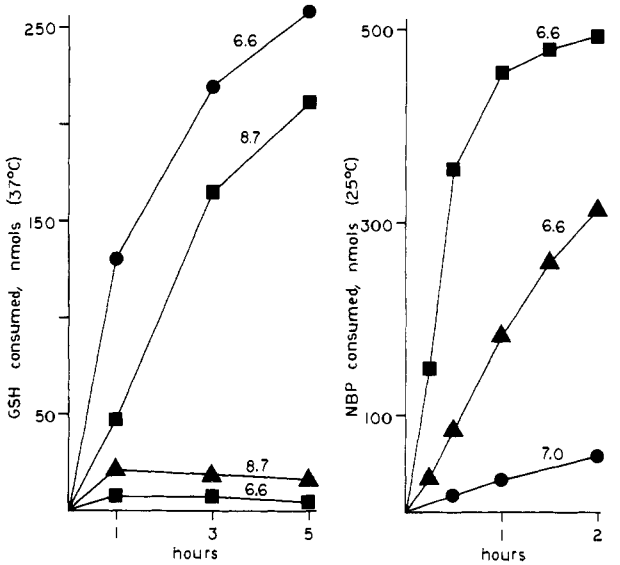


Figure 1. Consumption of glutathione (GSH) and 4-(*p*-nitrobenzyl)pyridine (NBP) in the presence of (diethylamino)ethyl chloride (●), **4** (▲), and **5** (■). Incubation mixtures initially contained 500 nmol of these nucleophiles at the indicated pH values. Glutathione and NBP adducts were determined in aliquots of reaction solutions by adaptation of published methods[9] as described in detail in the Experimental Section.

Reaction of 4 and 5 with Nucleophiles. Previously, **4** and **5** were found to react quantitatively with 4-(*p*-nitrobenzyl)pyridine (NBP) under "forcing" conditions, but **2** and **3** were inert.[6] Further studies are now reported on the reactivity of **4** and **5** toward NBP and glutathione (GSH) under conditions chosen to obtain measurable reaction rates and adequate solubility of all reactants. We

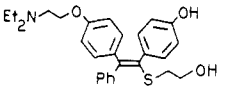
(6) Ruenitz, P. C.; Arrendale, R. F.; George, G. D.; Thompson, C. B.; Mokler, C. M.; Nanavati, N. T. *Cancer Res.* **1987**, *47*, 4015–4019.
(7) Ruenitz, P. C.; Bagley, J. R.; Nanavati, N. T. *J. Med. Chem.* **1988**, *31*, 1471–1475.
(8) (a) Coe, P. L.; Scriven, C. E. *J. Chem. Soc., Perkin Trans. 1* **1986**, 475–477. (b) Shani, J.; Gazit, A.; Livshitz, T.; Biran, S. *J. Med. Chem.* **1985**, *28*, 1504–1511. (c) McMurry, J. E.; Fleming, M. P.; Kees, K. L.; Krepski, L. R. *J. Org. Chem.* **1978**, *43*, 3255–3266.
(9) (a) Epstein, J.; Rosenthal, R. W.; Ess, R. J. *Anal. Chem.* **1955**, *27*, 1435–1439. (b) Friedman, O. M.; Boger, E. *Anal. Chem.* **1961**, *33*, 906–910.
(10) (a) Ruenitz, P. C.; Bagley, J. R.; Mokler, C. M. *J. Med. Chem.* **1982**, *25*, 1056–1060. (b) Ruenitz, P. C.; Bagley, J. R. *Biochem. Pharmacol.* **1985**, *34*, 2807–2809.
(11) (a) Sutherland, R. L.; Hall, R. E.; Taylor, I. W. *Cancer Res.* **1983**, *43*, 3998–4006. (b) Reddel, R. R.; Murphy, L. C.; Hall, R. E.; Sutherland, R. L. *Cancer Res.* **1985**, *45*, 1525–1531.

Table I. Interaction of 3 and 5 with Estrogen Receptors (ER) and Antiestrogen Binding Sites (AEBS)[a]

no.	IC_{50},[b] M (RBA[c]): affinity for ER	AEBS
3	7.8×10^{-7} (0.4)	2.2×10^{-8} (55)
5	3.0×10^{-6} (0.1)	3.2×10^{-8} (38)
estradiol	3.0×10^{-9} (100)	
tamoxifen		1.2×10^{-8} (100)

[a] MCF 7 cells were homogenized and fractions containing ER and AEBS were separated by centrifugation. Competitive binding assays were performed in triplicate at 0 °C as described,[10] with [^{3}H]estradiol and [^{3}H]tamoxifen as ligands for ER and AEBS, respectively. [b] Concentration of test compound which displaced 50% of specifically bound [^{3}H]estradiol or [^{3}H]tamoxifen. [c] IC_{50} of estradiol or tamoxifen divided by IC_{50} of the test compound times 100.

found (Figure 1) that **5** reacted readily with each of these reagents at rates comparable to those of a standard latent electrophile, 2-(diethylamino)ethyl chloride. While **4** did not react with GSH at 37 °C, incubation of a methanol solution of **4** with 2-mercaptoethanol at 60 °C for 4 h resulted in quantitative conversion to a single product as indicated by TLC. Its mass spectrum showed that displacement of chloro with a (2-hydroxyethyl)thio substituent had occurred. Its structure (**13**) was tentatively assigned by comparison of its ^{1}H NMR spectrum with those of related aryl and vinyl thioethers (see Discussion).



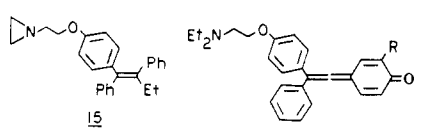
13

Interaction of 3 and 5 with Binding Sites from MCF 7 Cells. Compound **5** and its positional isomer **3** were good ligands for antiestrogen binding sites (AEBS) but were less effective in regard to their affinity for ER (Table I). Prolonged incubation of **5** in the presence of cytosol or 9000 g of supernatant from cell homogenates, under conditions similar to those in which **5** reacted with GSH, did not cause a time-dependent loss of specific binding capacity.

Suppression of MCF 7 Cell Proliferation by 1–5. All of the analogues of **1** were effective inhibitors in the 1–10 μM concentration range (Figure 2). Compounds **2** and **3** were more effective than respective positional isomers **4** and **5**.

Discussion

Previously, hydroxyclomiphenes **4** and **5**, but *not* **2** and **3**, were found to react with NBP at 90–95 °C in aqueous buffered solution.[6] This was suggested to be due to the ability of **4** and **5** to undergo conversion to electrophilic allene-quinones **14**. The present results (Figure 1) reconfirm these findings and indicate that conversion to postulated electrophile **14** proceeds under mild conditions.



15

14a, R = H
b, R = OMe

In order to study the point of attack of nucleophiles on **14**, **4** was allowed to react with excess 2-mercaptoethanol. The ^{1}H NMR spectrum of the product had features similar to those of other triarylethylenes in this study, plus resonances due to the presence of SCH_2CH_2OH. Specifically, SCH_2 and OCH_2 of this moiety appeared as higher order multiplets centered in turn at 2.49 ppm and 3.56 ppm.

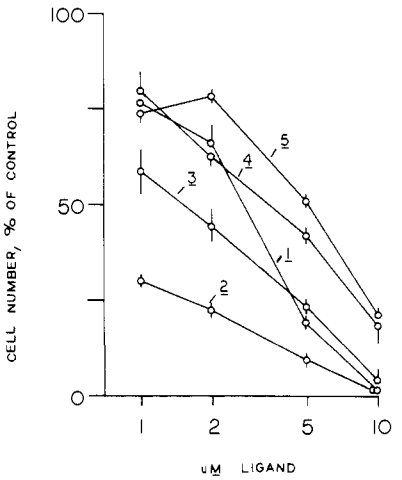


Figure 2. Inhibition of MCF 7 breast cancer cell proliferation by **1** and hydroxylated analogues. Cells (1×10^5) in exponential growth phase were plated into 25-cm^2 tissue culture flasks in 5 mL of RPMI 1640 culture medium supplemented with 10% fetal calf serum and other additives.[11] When cell numbers had reached ($2–2.5 \times 10^5$)/flask (ca. 72 h later), culture medium was changed and varying concentrations of drug were added as solutions in 5-μL aliquots of ethanol. Cell numbers were determined 5 days later, after ca. four population doublings, as previously described.[11] Data shown are expressed as percentages of cell numbers in control flasks and are means ±SEM of three to six estimates from one to two separate experiments.

Vinyl thioethers feature SCH_2 signals at 2.4–2.6 ppm, while in aryl thioethers, these signals are seen at 2.8–3.0 ppm.[12] Thus, in the spectrum of *p*-tolyl-β-hydroxyethyl thioether, SCH_2 was centered at 2.89 ppm, but in 1-(ethylthio)-1-(dimethylamino)ethylene and 1-(ethylthio)-2-methylcyclohexene these signals were at 2.40 and 2.56 ppm, respectively.[12b,c] This evidence suggests that 2-mercaptoethanol reacts specifically with the allenic carbon of **14** to afford **13**, in preference to conjugate addition involving one of the carbons of the quinone ring.

Since **5** was more reactive than **4** toward NBP and GSH (Figure 1), we wanted to find out whether this ligand interacted irreversibly with high affinity binding sites from MCF 7 human breast cancer cells. Affinities of **5** and its nonelectrophilic regioisomer (**3**) for ER (Table I) were similar to that of **1**[3a] but lower than that of **2**, which had an RBA of 90.[5b] This finding, incidentally, underscores the importance of the unobstructed hydroxyl group in **2** as a point of interaction of this ligand type with ER. The RBA's of **3** and **5** for MCF 7 cell AEBS approached that of tamoxifen. However, further experiments indicated that these compounds interacted only in reversible ways with AEBS and ER. This suggested that either **5** failed to undergo conversion to **14b** after binding or that ER and AEBS do not have nucleophilic groups of appropriate proximity to interact covalently with the reactive center of **14b**. The ligand binding site of ER is known to have at least one nucleophilic group since **15**, a ligand with an electrophilic aziridine ring in its side chain, reacted covalently at this site.[13]

The breast cancer cell-suppressive effects of a few ER ligands bearing alkylating moieties have been studied.[14]

(12) (a) Chamberlain, N. F. *The Practice of NMR Spectroscopy*; Plenum: New York, 1974; p 267. (b) Mukaiyama, T.; Aizawa, S.; Yamaguchi, T. *Bull. Chem. Soc. Jpn.* **1967**, *40*, 2641–2644. (c) Akiyama, F. *Bull. Chem. Soc. Jpn.* **1977**, *50*, 936–938.
(13) Katzenellenbogen, J. A.; Carlson, K. E.; Heiman, D. F.; Robertson, D. W.; Wei, L. L.; Katzenellenbogen, B. S. *J. Biol. Chem.* **1983**, *258*, 3487–3495.

For example, 2-[2-(mesyloxy)ethoxy]estrone reduced by 60% the growth of MCF 7 cells at a concentration of 10^{-8} M. No inhibitory effects were seen in ER-negative Evsa-T cells at a concentration of 10^{-6} M.[14] Also, **15** was an effective inhibitor of MCF 7 cell proliferation; its effect was similar to that of tamoxifen.[15] Results of the present study showed that **4** and **5** were not cytotoxic at concentrations up to 10 μM (Figure 2) and that antiproliferative potencies were somewhat less than those of nonelectrophilic hydroxyclomiphenes **2** and **3** at the concentrations tested. These results, together with our finding that **5** interacted reversibly but not irreversibly with ER, suggest that **4** and **5** inhibit MCF 7 cell proliferation by reversible interactions with receptors, similar to those of **1** and analogous triarylethylenes.[16]

The hydroxyclomiphenes **2–5** have been characterized as metabolites of **1** in experimental studies.[5a,6] The results summarized in Figure 2 indicate that **2–5** could contribute to the antiestrogenic effects of **1** if they are produced in sufficient amounts in vivo.

Experimental Section

All reactions were run under dry nitrogen. Progress of reactions and purity of products was analyzed by thin layer chromatography (TLC) using plastic strips coated with 0.2 mm silica gel 60 F 254 (EM Reagents) and chloroform–methanol–28% aqueous NH_3 (90–10–0.5, by volume) as developing solvent, unless indicated otherwise. Progress of chlorination reactions was checked by running Beilstein tests on aliquots of reaction solutions, since R_f values of **3** acetate and **12a,b** were similar to those of respective nonchlorinated precursors. Column chromatography was carried out by using EM Reagents Kieselgel 60 (230–400 mesh). Preparative TLC was run on 20 × 20 cm glass plates coated with 1 mm of silica gel GF 254 (Analtech) using the developing solvent described above. Fast atom bombardment mass spectrometry (FAB) was carried out as previously reported,[17] as were electron impact (70 eV) mass spectrometry (MS) and gas chromatography–mass spectrometry (GC-MS).[6]

Proton nuclear magnetic resonance spectra (^{1}H NMR) were recorded at 90 MHz (except for the product of reaction of **4** with 2-mercaptoethanol, which was recorded at 250 MHz) using $CDCl_3$ as solvent and Me_4Si as internal reference.

Starting Materials. [4-[2-(Diethylamino)ethoxy]phenyl]-magnesium bromide (**6**) was prepared as previously reported.[5b] Benzophenone **10** was prepared by alkylation of *p*-hydroxybenzophenone with 2-(diethylamino)ethyl chloride hydrochloride by a standard procedure:[10a] bp 172–174 °C (0.01 mmHg) (lit.[18] bp 200–204 °C (0.7 mmHg). The preparation of **2** has been reported.[19]

1-[(2-Methoxyethoxy)methoxy]-2-methoxy-4-cyanobenzene (7). A solution of 2.98 g (20 mmol) of 2-methoxy-4-cyanophenol in 10 mL of tetrahydrofuran (THF) was added to a stirred suspension of 0.58 g (24 mmol) of NaH in 2 mL of THF, at 0 °C. After gas evolution had ceased, a solution of 2.49 g (20 mmol) of 2-(methoxyethoxy)methyl chloride in 8 mL of THF was added. The mixture was allowed to warm to room temperature. After 16 h, the mixture was filtered. The filtrate was diluted with 20 mL of ether and the resulting solution was extracted serially with two 20-mL portions of 10% aqueous NaOH and then once with 25 mL of water. Then it was dried (Na_2SO_4), filtered, and concentrated in vacuo during which time the product crystallized. Recrystallization from ethanol–ether afforded 4.05 g (85%) of white crystals: TLC (CH_2Cl_2 solvent) one spot, R_f 0.85; IR (CCl_4) ν 2230 cm^{-1} (C≡N).

3-Methoxy-4-(benzyloxy)benzaldehyde (11b). To a suspension of 0.94 g (39 mmol) of NaH in 15 mL of stirred dimethylformamide (DMF) was added a solution of 5.5 g (36 mmol) of 3-methoxy-4-hydroxybenzaldehyde in 10 mL of DMF. After gas evolution ceased, 11 g (87 mmol) of benzyl chloride was added. The mixture was heated at 125 °C for 4 h. Then it was cooled and filtered. The filtrate was concentrated in vacuo. The residue was triturated with hexanes to furnish 7.3 g (83%) of a white solid, which separated from ethanol as white crystals: mp 62 °C; ^{1}H NMR δ 3.90 (s, 3, OCH_3), 5.20 (s, 2, OCH_2), 9.85 (s, 1, CHO).

4-(Benzyloxy)benzaldehyde (11a). This was prepared in exactly the same manner as was **11b**. The product crystallized from ethanol: mp 76 °C; ^{1}H NMR δ 5.15 (s, 2, OCH_2), 9.90 (s, 1, CHO).

4-[2-(Diethylamino)ethoxy]-3′-methoxy-4′-[(2-methoxyethoxy)methoxy]benzophenone (8). To a 0.6 M solution of **6** in 14 mL of THF was added dropwise a solution of 1.97 g (8.3 mmol) of **7** in 10 mL of THF. The solution was stirred and refluxed for 5 h. Then 6 mL of methanol was added dropwise. The mixture was concentrated in vacuo. The residue was suspended in 40 mL of methanol and centrifuged. To the supernatant was added 4 mL of saturated aqueous NH_4Cl. After 2 days at room temperature, the solution was concentrated. The residue was shaken with 20 mL of ether and 20 mL of 10% aqueous NaOH. The ether phase was extracted with 30 mL of 10% aqueous acetic acid. This aqueous extract was made basic by addition of 10% aqueous NaOH and was extracted with two 30-mL portions of ether. The combined ethereal extracts were dried (Na_2SO_4), filtered and concentrated in vacuo to give 0.73 g (20%) of a gold liquid: IR (neat) ν 1650 cm^{-1} (C=O); TLC (benzene–triethylamine, 90–10, v/v) one spot, R_f 0.62.

1-[4-[2-(Diethylamino)ethoxy]phenyl]-1-(3-methoxy-4-hydroxyphenyl)-2-phenylethene (9). A solution of 0.5 g (1.16 mmol) of **8** in 3 mL of ether was added dropwise to a stirred suspension of 0.7 g (4.64 mmol) of benzylmagnesium chloride in 4 mL of ether. After 2 h, excess benzylmagnesium chloride was hydrolyzed by addition of saturated aqueous NH_4Cl. The mixture was filtered and the filtrate was washed with 10 mL of ether. The combined filtrates were extracted with 5 mL of 5% aqueous HCl. After 48 h at room temperature, this solution was washed with 10 mL of ether, and **9**·HCl was extracted with two 10-mL portions of $CHCl_3$. The combined extracts were dried (Na_2SO_4), filtered, and concentrated in vacuo. The product was converted to the free base by partitioning it between 15 mL of ether and 10 mL of 1% aqueous Na_2CO_3. The ether extract was dried (Na_2SO_4), filtered, and concentrated in vacuo to give 0.25 g (52%) of a light yellow oil: TLC (chloroform–methanol–28% aqueous NH_3, 95–5–0.5, v/v) one spot, R_f 0.38; IR (neat) ν 3430 cm^{-1} (broad, OH stretch); MS, m/z (rel intensity) 417 ($M^{\cdot+}$, 4), 402 (2, M – CH_3), 318 (4), 86 (100). This product was used without further characterization.

1-[4-[2-(Diethylamino)ethoxy]phenyl]-1-(3-methoxy-4-hydroxyphenyl)-2-phenyl-2-chloroethene (3). To 0.25 g (0.6 mmol) of **9** was added 1.5 mL of acetic anhydride. The solution was stirred at room temperature for 6 h and concentrated in vacuo. The residue was dissolved in 10 mL of chloroform. The solution was washed successively with 5-mL portions of 5% aqueous $NaHCO_3$, water, and 5% aqueous HCl. The solution was dried (Na_2SO_4) and filtered. To the filtrate was added 0.095 g (0.7 mmol) of *N*-chlorosuccinimide (NCS). The mixture was stirred and refluxed for 24 h. The mixture was concentrated in vacuo. The residue was shaken with 10 mL of ether and 10 mL of 5% aqueous $NaHCO_3$. The organic phase was concentrated in vacuo; the residue was dissolved in 4 mL of methanol and 1 mL of 10% aqueous HCl was added. The solution was heated at 70 °C for 10 min and then concentrated in vacuo. The residue was subjected to preparative TLC. The major component, R_f 0.50–0.55, was removed and eluted with 15 mL of ethanol. The mixture was

(14) LeClercq, G.; Devleeschouwer, N.; Heuson, J. C. *J. Steroid Biochem.* **1983**, *19*, 75–85.
(15) Wei, L. L.; Mangel, W. F.; Katzenellenbogen, B. S. *J. Steroid Biochem.* **1985**, *23*, 875–881.
(16) (a) Sutherland, R. L.; Murphy, L. C. *Mol. Cell. Endocrinol.* **1982**, *25*, 5–23. (b) Wakeling, A. E.; Valcaccia, B.; Newboult, E.; Green, L. R. *J. Steroid Biochem.* **1984**, *20*, 111–120. (c) Rochefort, H.; Borgna, J. L.; Evans, E. *J. Steroid Biochem.* **1983**, *19*, 69–74. (d) Sutherland, R. L.; Watts, C. K. W.; Hall, R. E.; Ruenitz, P. C. *J. Steroid Biochem.* **1987**, *27*, 891–897.
(17) Martin, S. A.; Costello, C. E.; Biemann, K. *Anal. Chem.* **1982**, *54*, 2362–2368.
(18) Allen, R. E.; Palopoli, F. P.; Schumann, E. L.; Van Campen, M. G., Jr. U.S. Patent 2 914 561-4, 1959; *Chem. Abstr.* **1960**, *54*, 5581e–5584e.
(19) Ruenitz, P. C. *Drug. Metab. Dispos.* **1981**, *9*, 456–460.

filtered and concentrated in vacuo to give 0.12 g (44%) of a yellow gum. This was dissolved in 0.5 mL of 2-butanone and a solution of 56 mg of citric acid monohydrate in 0.5 mL of 2-butanone was added. Storage at 8 °C afforded 54 mg of light yellow crystals, mp 96–98 °C. Anal. ($C_{33}H_{38}ClNO_{10}$) C, H, Cl, N. A small portion of this was converted to the free base as described above: ^{1}H NMR δ 0.85–1.10 (m, 6, NCH_2CH_3), 2.28–2.61 (m, 4, CH_2CH_3), 2.70–2.97 (m, 2, NCH_2CH_2O), 3.47 and 3.84 (s, 1.5 each, OCH_3), 3.91–4.08 (m, 2, CH_2O), 6.65–7.59 (m, 12, Ar H); FAB, m/z 452.2007 (MH^+), calcd for $C_{27}H_{30}ClNO_3H^+$ 452.1992; CIMS (isobutane) m/z 452 (MH^+) (rel intensity) 100; GC/MS (TMS ether) retention time of component with $M^{\cdot+}$ = m/z 523: 22.6 min, estimated purity = 98%.

1-[4-[2-(Diethylamino)ethoxy]phenyl]-1-phenyl-2-[4-(benzyloxy)phenyl]-2-chloroethene (12a). Titanium(IV) chloride (3.44 g, 18 mmol) was added dropwise to a stirred suspension of zinc powder (2.3 g, 36 mg atom) in 30 mL of THF at −10 °C under nitrogen. Then the mixture was warmed to room temperature and refluxed for 2 h. After being cooled to room temperature, a solution of 0.92 g (3.1 mmol) of **10** and 0.7 g (3.0 mmol) of **11a** in 30 mL of THF was added dropwise with stirring. After completion of addition, the reaction mixture was refluxed for 2 h, cooled, and poured into 300 mL of 10% aqueous potassium carbonate with stirring. The mixture was serially extracted with three 150-mL portions of ether. The combined ether extracts were washed with 300 mL of water and dried over anhydrous Na_2SO_4. Evaporation of solvent left 0.9 g of a yellow gum. This was chromatographed on 25 g of silica gel. Elution with 200 mL of chloroform and then with 150 mL of 2% methanol in chloroform gave 0.72 g (49%) of a colorless oil: ^{1}H NMR δ 1.03 and 1.07 (t, 6, CH_3), 2.40–3.00 (m, 6, NCH_2), 3.95 and 4.05 (t, 2, OCH_2), 4.97 (s, 2, CH_2Ph), 6.45–7.50 (m, 18, Ar H); MS, m/z (rel intensity) 386 (1, M − CH_2Ph) 91 (75, CH_2Ph), 86 (100, Et_2NCH_2); TLC, one spot, R_f 0.72. To an ethereal solution of 0.1 g (0.02 mmol) of this was added excess ethereal HCl. The precipitate was dissolved in dry chloroform (5 mL) and 30 mg (0.22 mmol) of *N*-chlorosuccinimide was added. The mixture was refluxed for 3 h and then concentrated in vacuo. The residue was dissolved in 5 mL of ether and the solution was washed with 5 mL of 10% aqueous Na_2CO_3 followed by three 5-mL portions of H_2O. The ethereal solution was dried (Na_2SO_4) and concentrated to give 70 mg (65%) of a yellow oil. This was dissolved in 2 mL of 2-butanone containing 85 mg of citric acid. Dilution with 2 mL of ether and storage at 8 °C gave 65% mg of white crystals, which were recrystallized once from 2-butanone: mp 90–94 °C. Anal. ($C_{39}H_{42}ClNO_9 \cdot 0.75H_2O$) C, H, Cl, N.

1-[4-[2-(Diethylamino)ethoxy]phenyl]-1-phenyl-2-chloro-2-[3-methoxy-4-(benzyloxy)phenyl]ethene (12b). The deschloro intermediate was prepared as described above starting with 3.44 g (18 mmol) of $TiCl_4$, 2.3 g (36 mg atom) of Zn powder, 0.92 g (3.1 mmol) of **10**, and 0.73 g (3.0 mmol) of **11b**. Chromatography gave 0.39 g (36%) of yellow solid: TLC, R_f 0.69; ^{1}H NMR δ 1.08 (t, 6, CH_3), 2.70 (q, 4, NCH_2CH_3), 2.90 (t, 2, NCH_2CH_2), 3.75 (s, 3, OCH_3), 5.10 (s, 2, OCH_2Ph), 6.50–7.25 (m, 17, Ar H). Chlorination of 100 mg of this was carried out as described for synthesis of **12a** to give 80 mg (75%) of yellow oil. This was dissolved in 1 mL of 2-butanone containing 35 mg of citric acid. The product separated as white crystals, 65 mg: mp 100–105 °C. Anal. ($C_{40}H_{44}ClNO_{10} \cdot H_2O$) C, H, Cl, N.

Debenzylation of 12a,b. General Procedure. To a solution of 0.1 mmol of **12a** or **12b** in 1.5 mL of glacial acetic acid was added 15 mg of 10% Pd on charcoal. The mixture was shaken under 20 psi of H_2 at ambient temperature for 2 h. Then the mixture was filtered and the filtrate was freeze-dried. The product was dissolved in 1 mL of 2-butanone, which contained 19 mg (0.1 mmol) of citric acid. The solution was diluted to turbidity with ether and kept at 8 °C overnight. The product separated as white crystals. By this procedure **12a** was converted to 35 mg (57%) of 4 citrate, mp 90–92 °C. Anal. ($C_{32}H_{36}ClNO_9$) C, H, Cl, N. Equilibration of 5 mg of this between 1 mL of ether and 1 mL of 10% aqueous Na_2CO_3 gave **4** as a colorless oil: MS, m/z (rel intensity) 421 (2, $M^{\cdot+}$), 86 (100, Et_2NCH_2); GC MS (TMS ether) retention time of component with $M^{\cdot+}$ = m/z 493: 25.8 min. Similarly, **12b** afforded 44 mg (68%) of **5** citrate hemihydrate, mp 92–96 °C. Anal. ($C_{33}H_{38}ClNO_{10.5}$) C, H, Cl. The free base (**5**) was prepared as described above: MS, m/z (rel intensity) 451 (2, $M^{\cdot+}$), 86 (Et_2NCH_2, 100). FAB, m/z 452.1997 (MH^+), calcd for $C_{27}H_{30}ClNO_3H^+$ 452.1992.

Reactions of 4 and 5 with Nucleophiles. A. Glutathione. To a solution of 0.5 μmol of the test compound in 100 μL of methanol were added 0.5 μmol of glutathione in 50 μL of H_2O and 50 μL of 200 mM buffer (Tris, pH 7.4, or glycine, pH 9.6). Note: these buffers also contained 10 mM EDTA. Control reactions each containing 50 μL of the appropriate buffer, 100 μL of methanol, and either 50 μL of H_2O or 0.5 μmol of glutathione in 50 μL of H_2O were run simultaneously. Solutions were warmed to 37 °C and the pH of each was determined with a microelectrode. Then each was capped and allowed to stand at 37 °C. At selected time intervals, solutions were vortexed and 40 μL of each was mixed with 940 μL of 0.2 M potassium phosphate buffer, pH 7.4, 20 μL of 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) in methanol and 1.0 mL of methanol. Instantaneous consumption of glutathione was accompanied by formation of an equimolar amount of 5-mercapto-2-nitrobenzoic acid,[20] found under these conditions to have ϵ_{412} of 16.0 mM^{-1} cm^{-1}. Determination of absorbance at 412 nm was thus used to calculate glutathione levels in reactions run in the absence and presence of the test compound.

B. 4-(*p*-Nitrobenzyl)pyridine (NBP). To a solution of 0.5 μmol of the test compound in 0.1 mL of methanol was added 1.5 mL of 200 mM buffer (see above). The solution was mixed and cooled to 0 °C, and 0.4 mL of 0.5% NBP (9.3 μmol) in methanol was added. The solution was warmed quickly to 23 °C. The pH was determined with a microelectrode. The solution was analyzed at selected times after preparation as follows. An aliquot (0.25 mL) of the vortexed reaction solution was diluted with 0.2 mL of acetone and 0.2 mL of 1 N aqueous NaOH. Then 1.0 mL of ethyl acetate was added. The mixture was vortexed and after the phases had separated, absorbance of the upper, magenta-colored layer was determined at 540 nm. A parallel reaction, from which the test compound had been omitted, was prepared and analyzed as a control. The amount of N-derivatized NBP present was calculated by using ϵ_{540} of 14.0 mM^{-1} cm^{-1}.

C. 2-Mercaptoethanol. To 1.1 mg (2.6 μmol) of 4 was added 0.2 mL of methanol containing 7.8 mg (100 μmol) of 2-mercaptoethanol. The solution was heated at 60 °C for 4 h. The solution was concentrated under a stream of N_2. The product was purified by preparative TLC to give 1.1 mg (91%) of a white solid: UV (MeOH) λ_{max} 315 nm; MS, m/z 463 ($M^{\cdot+}$, 0.4) 100 (30, $Et_2NCH_2CH_2$) 86 (100, Et_2NCH_2); ^{1}H NMR (250 MHz) δ 1.06 and 1.10 (t, 6, CH_3), 1.71 (s, $W_{1/2}$ = 34 Hz, 2, OH), 2.48 and 2.50 (t, 2, SCH_2), 2.62 (m, 4, NCH_2CH_3), 2.82 (t, 2, NCH_2CH_2), 3.55 and 3.56 (t, 2, CH_2OH), 3.97 and 4.13 (t, 2, CH_2OAr), 6.5–7.5 (m, 13, vinyl and Ar H).

Interaction of 3 and 5 with High Affinity Binding Sites from MCF 7 Human Breast Cancer Cells. A. Preparation of Subcellular Fractions. Cells were maintained by weekly passage in RPMI 1640 medium supplemented with 10% v/v fetal calf serum and other additives as described previously.[11] Cells used for binding studies were obtained by inoculating 3×10^6 logarithmically growing cells into 150-cm^2 flasks, in 50 mL of medium. Medium was changed 3 days and 5 days after inoculation and cells were harvested on day 7, when cell numbers had reached $(3–4 \times 10^7)$/flask, using 1 mM EDTA in Dulbecco's phosphate buffered saline (PBS). Cells were washed once with PBS and once with 10 mM Tris buffer, pH 7.4, containing 1 mM EDTA and 1 M NaCl, each wash being followed by centrifugation at 800*g* for 10 min. Pelleted cells were stored at −17 °C overnight. Thawed pellets were resuspended in ice-cold Tris–EDTA–NaCl buffer (above) at 3×10^7 cells/mL. After 30 min, suspensions were homogenized with a Teflon-glass homogenizer. Centrifugation of the homogenate at 9000*g* for 20 min (4 °C) afforded the supernatant used for antiestrogen binding site studies. Alternatively, the homogenate was treated with two volumes of dextran-coated charcoal suspension (0.1% dextran, 1% activated charcoal in 10 mM Tris buffer, pH 7.4, containing 1.5 mM EDTA and 3 mM NaN_3) at 0 °C for 1 h. The mixture was centrifuged at 450*g* for 10 min, and the resulting supernatant was centrifuged at 105000*g* for 1 h to afford the cytosol used in estrogen receptor binding studies.

(20) Flavin, M. *J. Biol. Chem.* **1962**, *237*, 768–777.

B. Competitive Binding Assays. Aliquots (0.2 mL) of cytosol or 9000*g* supernatant were incubated, in turn, with 3.0 nM [^{3}H]estradiol or 1.4 nM [^{3}H]tamoxifen and increasing concentrations of test ligands. The extent of specific binding of ^{3}H ligands was determined as described previously,[10] and data were plotted as percentage of ^{3}H ligand bound as a function of the log of test ligand concentration.

C. Pretreatment of MCF 7 Subcellular Fractions with 5. To 6 mL of 9000*g* supernatant from 1×10^7 MCF 7 cells (see A) was added sufficient 0.2 M glycine buffer, pH 9.6, and 0.2 M Tris buffer, pH 7.4 (each buffer contained 10 mM EDTA), to give a final volume of ca. 12 mL with a pH of 8.7 (37 °C). To a 1.0-mL aliquot of this (found in preliminary experiments to contain ca. 770 fmol/mL of specific [^{3}H]tamoxifen binding sites) was added 0.4 nmol of **5** citrate in 20 μL of ethanol. After 3 h at 37 °C, the mixture was cooled to 0 °C. To 0.5 mL of the suspension was added 1.5 mL of dextran-coated charcoal. After 15 min, the mixture was centrifuged at 400*g* for 10 min. Aliquots (0.2 mL) of the supernatant were used for determination of total and nonspecific binding of [^{3}H]tamoxifen using established methods.[10] A control experiment was run exactly as described except that **5** citrate was added to the 1.0-mL aliquot just prior to addition of dextran-coated charcoal. Experiments with cytosol were carried out in exactly the same way at 25 °C, using [^{3}H]estradiol as the radioligand.

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Registry No. **3**, 117095-59-7; **3**·citrate, 117095-70-2; **4**, 117095-64-4; **4**·citrate, 117095-66-6; **5**, 117095-65-5; **5**·citrate, 117095-67-7; **6**, 35258-26-5; **7**, 117095-56-4; **8**, 117095-57-5; **9**, 117095-58-6; **10**, 796-77-0; **11a**, 4397-53-9; **11b**, 2426-87-1; **12a**, 117095-60-0; **12a**·citrate, 117120-05-5; **12b**, 117095-61-1; **12b**·citrate, 117095-69-9; **13**, 117095-68-8; $PhCH_2MgCl$, 6921-34-2; 2-methoxy-4-cyanophenol, 4421-08-3; 3-methoxy-4-hydroxybenzaldehyde, 121-33-5; *p*-hydroxybenzaldehyde, 123-08-0; 1-[4-[2-(diethylamino)ethoxy]phenyl]-1-phenyl-2-[(4-benzyloxy)phenyl]-2-hydroxyethene, 117095-62-2; 1-[4-[2-(diethylamino)ethoxy]-phenyl]-1-phenyl-2-hydroxy-2-[3-methoxy-4-(benzyloxy)-phenyl]ethene, 117095-63-3.

Synthesis and Biological Evaluation of a Monocyclic, Fully Functional Analogue of Compactin

Clayton H. Heathcock,* Brian R. Davis, and Cheri R. Hadley

Department of Chemistry, University of California, Berkeley, California 94720. *Received January 19, 1988*

Compound **8**, a monocyclic analogue of compactin, has been prepared and its efficacy as an inhibitor of 3-hydroxy-3-methylglutarylcoenzyme A reductase (HMGR) evaluated. The synthesis (Schemes I and II) requires seven steps starting with di-(−)-menthyl fumarate (**9**) and employs the useful *RR*-phosphonate reagent **14** to attach the mevinic acid side chain to aldehyde **13**. A molecular mechanics study shows that the preferred conformations of **18** (a model for compactin) and **19** (a model for **8**) are nearly identical. Compound **8** inhibits HMGR with IC_{50} = 320 μM, compared to a corresponding value of 32 nM for the compactin ketone, **5**. The factor of 10 000 difference in the two inhibitors corresponds to a difference in binding energy of 5.45 kcal mol^{-1}, or 1.36 kcal mol^{-1} for each of the four carbons of **5** that are missing in analogue **8**. This quantitative difference is consistent with the idea that the decalin moiety of the mevinic acids plays a purely hydrophobic role in binding the inhibitors to the enzyme.

The mevinic acids compactin (**1**) and mevinolin (**2**) have been shown to be effective in lowering plasma cholesterol levels in clinical trials,[1-4] and it is clear that these and related compounds will have an important pharmacological use as hypocholesterolemic agents.[5] Compounds **1** and **2** function by inhibiting the enzyme 3-hydroxy-3-methylglutarylcoenzyme A reductase (HMGR) in its conversion of HMG CoA to mevalonate and coenzyme A. It has been shown that the effective inhibitors are the 3-(*R*),5(*R*)-dihydroxy acids **3** and **4**. Recent work has confirmed the importance of the 3*R* stereochemistry and has shown that the 5-keto analogues have inhibitory activity that is indistinguishable within experimental error from that of the parent dihydroxy acid.[6] Thus, the 5-keto compounds **5**,[6] **6**,[6] and **7**[7] have IC_{50} values of 32 nM, 1 nM, and 1.3 mM, respectively, compared with IC_{50} values of 13 nM, 1.6 nM, and 0.6 mM for the corresponding 3-(*R*),5(*R*)-dihydroxy acids.

Compounds **1–6** are exceedingly potent competitive inhibitors of HMGR; their IC_{50} values are on the order of 10^{-4} of K_m for the natural substrate of the enzyme.[6,8] Available evidence shows that the decalinic moiety is responsible for much of this tight binding. This bicyclic hydrocarbon unit might provide hydrophobic binding, or

(1) (a) Shigeinatsu, H.; Hata, Y.; Yamamoto, M.; Oikawa, T.; Yamauchi, Y.; Nakaya, N.; Goto, Y. *Geriatr. Med.* **1979**, *17*, 1564. (b) Tobert, J. A.; Bell, G. D.; Birtwell, J.; James, I.; Kokovetz, W. R.; Pryor, J. S.; Buntinx, A.; Holmes, I. B.; Chao, Y. S.; Bologese, J. A. *J. Clin. Invest.* **1982**, *69*, 913. (c) Yamamoto, A.; Sudo, H.; Endo, A. *Atherosclerosis* **1980**, *35*, 259. (d) Mabuchi, H.; Haba, T.; Tatami, R.; Miyamoto, S.; Sakai, Y.; Wakasugi, T.; Watanabe, A.; Koizumi, J.; Takeda, R. *N. Engl. J. Med.* **1981**, *305*, 478. (e) Mabuchi, H.; Sakai, T.; Sakai, Y.; Yoshimura, A.; Watanabe, A.; Wakasugi, T.; Koizumi, J.; Takeda, R. *N. Engl. J. Med.* **1983**, *308*, 609.

(2) Havel, R. J.; Hunninghake, D. B.; Illingworth, D. R.; et al. *Circulation* **1985**, *72* (Suppl. 3), 198.

(3) Therapeutic Response to Lovastatin (Mevinolin) in Nonfamilial Hypercholesterolemia *JAMA, J. Am. Med. Assoc.* **1986**, *42*, 4909.

(4) (a) Nakaya, N.; Homma, Y.; Tamachi, H.; Goto, Y. *Atherosclerosis* **1986**, *61*, 125. (b) Alberts, A. W.; Chen, J.; Huff, J.; Hunt, V.; Kuron, G. *IXth Int. Symp. Drugs Affect. Lipid Metab. (Oct 22–25, Florence)* **1986**, 8. (c) Nakaya, N.; Goto, Y. *Ibid.* **1986**, 67.

(5) Mevinolin (generic name lovastatin) was recently approved by the Food and Drug Administration for treatment of hypercholesterolemia; the drug is being marketed by Merck Sharp & Dohme under the name Mevacor.

(6) Heathcock, C. H.; Hadley, C. R.; Rosen, T.; Theisen, P. D.; Hecker, S. J. *J. Med. Chem.* **1987**, *30*, 1858.

(7) Heathcock, C. H.; Theisen, P. C.; Hadley, C. R. Unpublished results.

(8) (a) Tanazawa, K.; Endo, A. *Eur. J. Biochem.* **1979**, *98*, 195. (b) Alberts, A. W.; Chen, J.; Kuron, G.; Hunt, V.; Huff, J.; Hoffman, G.; Rothrock, J.; Lopez, M.; Joshua, H.; Harris, E.; Patchett, A.; Monaghan, R.; Currie, S.; Stapley, E.; Albers-Schonberg, G.; Hensens, O.; Hirschfield, J.; Hoogstein, K.; Liesch, J.; Springer , J. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 3957.

0022-2623/89/1832-0197$01.50/0