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Hypocholesterolemic Agents IV: Inhibition of β-Hydroxy-β-methylglutaryl Coenzyme A Reductase by Arylalkenyl and Arylepoxy Hydrogen Succinates and Glutarates

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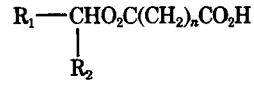
Abstract □ Two series of half acid esters of succinic and glutaric acids were synthesized and assayed for inhibition of rat liver β-hydroxy-β-methylglutaryl coenzyme A reductase. Irreversible inhibition was studied by incorporation of a potential alkylating group (the epoxide function) into the side chain of the alcohol portion of the half acid esters. Incorporation of a terminal olefin function into the side chain of the alcohol portion of the half acid esters provided a group that could form a charge–transfer complex. Neither irreversible inhibition nor formation of a charge–transfer complex was indicated from these studies; however, the two series of half acid esters exhibited reversible inhibition.

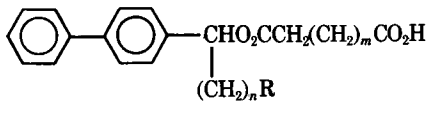
Keyphrases □ Hypocholesterolemic agents, potential—series of arylalkenyl and arylepoxy hydrogen succinates and glutarates, synthesis, effect on β-hydroxy-β-methylglutaryl coenzyme A reductase □ Enzymes—β-hydroxy-β-methylglutaryl coenzyme A reductase, inhibition by series of arylalkenyl and arylepoxy hydrogen succinates and glutarates □ Succinates, arylalkenyl and arylepoxy—substituted series, synthesis, effect on β-hydroxy-β-methylglutaryl coenzyme A reductase □ Glutarates, arylalkenyl and arylepoxy—substituted series, synthesis, effect on β-hydroxy-β-methylglutaryl coenzyme A reductase

Previous reports (1, 2) presented an approach to the design of inhibitors of the enzyme β-hydroxy-β-methylglutaryl coenzyme A reductase as potential hypocholesterolemic agents. These studies led to the development of reversible inhibitors (I) of yeast and rat liver β-hydroxy-β-methylglutaryl coenzyme A reductase. Structure–activity relationships indicated that maximum activity was observed when R_1 = biphenylyl, R_2 = *n*-butyl, and n = 1–4.

This study describes the synthesis and assay of potential irreversible inhibitors (II) of β-hydroxy-β-methylglutaryl coenzyme A reductase based upon I.



I: $n = 0$–6



II: R = —CH(O)CH$_2$, n = 1–3, m = 1 or 2

III: R = —CH=CH$_2$, n = 1–3, m = 1 or 2

The epoxide group was selected as the potential alkylating group on the basis of its relatively small size and its reactivity with nucleophiles.

The arylalkenylcarbinols (V), which were precursors of the arylepoxycarbinols (VI) used in the synthesis of the epoxy acid esters (II), were also esterified to give the corresponding alkenyl acid esters (III). Not only were these alkenyl acid esters readily available from a synthetic point of view, but they also could provide useful information when assayed as potential reversible inhibitors. The possibility exists that the terminal olefinic bond found in these alkenyl acid esters might act as an electron donor in the formation of a charge–transfer complex. Alkenes are well known for acting as donors in the formation of a charge–transfer complex because of their electron-rich π-clouds (3).

EXPERIMENTAL[1]

Chemistry—The previously described method (1) of preparing the arylalkylcarbinols was used with only slight modifications. Alkenyl bromides were utilized and the resulting arylalkenylcarbi-

[1] Melting points were determined with a Thomas-Hoover capillary melting-point apparatus and are uncorrected. Elemental analyses were carried out by Mr. Paul E. Marecki, Department of Pharmaceutical Chemistry, and by Galbraith Laboratories, Knoxville, Tenn. IR spectra were determined using a Perkin-Elmer model 237 spectrophotometer. NMR spectra were determined using a Perkin-Elmer model R-24 spectrometer or a Varian Associates A-60 spectrometer in deuterochloroform, using tetramethylsilane as the internal reference.

Table I—Properties of Arylalkenylcarbinols and Arylepoxycarbinols

Compound	n	Yield, %	Physical Description	Formula	Analysis, % Calc.	Found
V	1	69	63.5–65°[a,b]	$C_{16}H_{16}O$	C 85.7 H 7.2	86.0 7.2
V	2	94	68–68.5°[a,b]	$C_{17}H_{18}O$	C 85.7 H 7.6	85.6 7.7
V	3	46	55–58°[a,b]	$C_{18}H_{20}O$	C 85.7 H 8.0	85.5 7.6
VI	1	97	Colorless liquid[b,c]	$C_{16}H_{16}O_2$	C 80.0 H 6.7	79.8 6.5
VI	2	96	Colorless liquid[b,c]	$C_{17}H_{18}O_2$	C 80.3 H 7.1	—[d] —
VI	3	92	Beige liquid[b,e]	$C_{18}H_{20}O_2$	C 80.6 H 7.5	80.6 7.2

[a] White solid. [b] IR spectra (chloroform) and NMR spectra (deuterochloroform) were consistent with the assigned structures. An example of each is given in the *Experimental* section. [c] Purified by molecular distillation. [d] No satisfactory analytical data could be obtained. [e] No purification was carried out.

nols (V) were epoxidized using *m*-chloroperbenzoic acid (Scheme I). The arylepoxycarbinols (VI) were then treated with either succinic or glutaric anhydride, using a modification of the method of Steglich and Höfle (4). This method afforded the desired epoxy acid esters (II). The arylalkenylcarbinols also were treated with succinic or glutaric anhydride to afford the alkenyl acid esters (III).

Preparation of Arylalkenylcarbinols—The arylalkenylcarbinols listed in Table I were prepared by the treatment of 4-biphenylcarboxaldehyde[2] with the appropriate Grignard reagent. 1-(4-Biphenylyl)-3-buten-1-ol (V, n = 1) was prepared using the method of Tschesche and Machleidt (5). 1-(4-Biphenylyl)-4-penten-1-ol (V, n = 2) and 1-(4-biphenylyl)-5-hexen-1-ol (V, n = 3) were prepared using a procedure described previously (1).

Table I lists the pertinent data for these compounds. An example of typical IR and NMR spectra is given here for 1-(4-biphenylyl)-3-buten-1-ol; IR ($CHCl_3$): 3590 (medium, OH) and 1640 (medium >C=CH_2) cm^{-1}; NMR ($CDCl_3$): δ 2.47 (t, 2H, CH_2), 2.68 (s, 1H, OH), 4.65 (t, 1H, R_3CH), 4.87–5.27 (m, 2H, >C=CH_2), 5.48–6.18 (m, 1H, CH=), and 7.20–7.70 (m, 9H, aromatic) ppm.

Preparation of Arylepoxycarbinols—The arylepoxycarbinols listed in Table I (VI, n = 1–3) were prepared by the treatment of the appropriate arylalkenylcarbinol with *m*-chloroperbenzoic acid. A typical example is given here. The method of Fieser and Fieser (6) was used. To a solution of 3.36 g (0.015 mole) of 1-(4-biphenylyl)-3-buten-1-ol (V, n = 1) in 25 ml of chloroform was added, in a dropwise manner with stirring and cooling in an ice bath, a solution of 5.06 g (0.025 mole) of 85% *m*-chloroperbenzoic acid in 100 ml of chloroform. The mixture was stirred at 25° for 18 hr, and a solution of freshly prepared 10% sodium sulfite (75 ml) was added (negative starch iodide test).

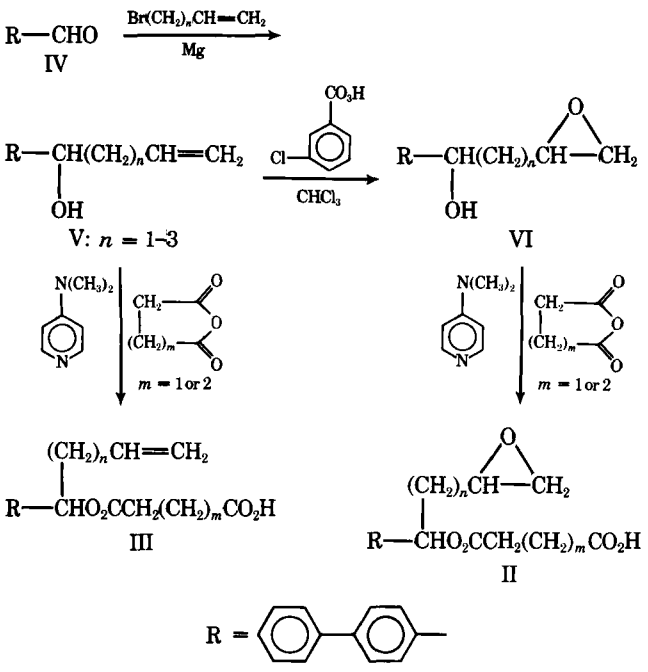
The organic phase was washed with 5% sodium bicarbonate, water, and then a saturated sodium chloride solution. The solution was dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure afforded 3.50 g (97%) of the arylepoxyalcohol (VI, n = 1) as a brown liquid. Molecular distillation using a Hickman still provided an analytical specimen as a colorless liquid; IR ($CHCl_3$): 3600 (medium, free OH), 3450 (medium, broad, hydrogen-bonded OH), and 835 (epoxide) cm^{-1}; NMR ($CDCl_3$): δ 1.90 (t, 2H, CH_2), 2.32–3.22 (m, 3H, $\overset{O}{\text{CH}}—CH_2$), 3.48 (s, 1H, OH), 4.84 (t, 1H, R_3CH), and 7.27–7.66 (m, 9H, aromatic) ppm.

Preparation of Alkenyl Acid Esters and Epoxy Acid Esters—The acid esters listed in Table II were prepared by a modification of the procedure of Steglich and Höfle (4) and Guyer *et al.* (2), except that the triethylamine was omitted and the reaction time was increased to 72 hr.

Assay of Enzyme Activity and Inhibition—Rat liver microsomes containing β-hydroxy-β-methylglutaryl coenzyme A reductase activity were prepared according to Shapiro and Rodwell (7). The assay procedure used was described previously (2). The enzyme incubation mixture consisted of $NADP^+$ (9.7 μmoles)[3], glucose 6-phosphate (59.7 μmoles)[3], torula yeast glucose 6-phosphate dehydrogenase (5 units)[3], microsomal protein (2 mg), 0.1 ml of ethylene glycol monoethyl ether containing the inhibitor, and sufficient assay buffer (30 m*M* edetic acid, 70 m*M* sodium chloride, and 10 m*M* β-mercaptoethanol, pH 6.8) to produce a total volume of 2.9 ml.

Enzymatic activity was initiated by the addition of 0.1 ml of a solution containing 94 nmoles (31 μ*M*) of 3-^{14}C-*dl*-β-hydroxy-β-methylglutaryl coenzyme A prepared (8) from 3-^{14}C-β-hydroxy-β-methylglutaric acid[4]. After 30 min of incubation, the reaction was stopped and 5-^{3}H-*dl*-mevalonolactone[4] was added. Mevalonolactone was extracted and isolated by TLC, and radioactivity was determined in a liquid scintillation counter[5].

Assay of Irreversible Enzyme Inhibition—The assay procedure used was that already described, except that the mixture was preincubated with the inhibitor at 37° for 20 min prior to the addition of the substrate. The inhibitor concentration used was that found to give the $(I/S)_{50\%}$ (Table III). Then 94 (31 μ*M*) or 517 (172 μ*M*) nmoles of the substrate was added.



Scheme I

[2] Aldrich Chemical Co.

[3] Sigma Chemical Co.

[4] New England Nuclear.

[5] Nuclear-Chicago Mark I.

Table II—Properties of Arylalkenyl and Arylepoxy Acid Esters

Compound	*n*	*m*	Yield, %	Melting Point (Salt)	Formula	Analysis, %[a] Calc.	Found
III*a*	1	1	79	143–144°[b]	$C_{28}H_{30}N_2O_4S \cdot \frac{1}{2}H_2O$	C 67.3 H 6.2 N 5.6	67.4 6.2 5.6
III*b*	2	1	57	118–119°[b]	$C_{29}H_{32}N_2O_4S$	C 69.0 H 6.4 N 5.6	68.8 6.4 5.6
III*c*	3	1	58	125–127°[b]	$C_{30}H_{34}N_2O_4S \cdot 1H_2O$	C 67.2 H 6.3 N 5.2	67.3 6.5 5.3
III*d*	1	2	26	146.5–147°[b]	$C_{29}H_{32}N_2O_4S$	C 69.0 H 6.4 N 5.6	69.2 6.5 5.5
III*e*	2	2	38	122–123°[c]	$C_{30}H_{34}N_2O_4S \cdot \frac{1}{2}H_2O$	C 68.3 H 6.4 N 5.3	68.4 6.5 5.4
III*f*	3	2	42	100–101.5°[b]	$C_{31}H_{36}N_2O_4S$	C 68.7 H 6.6 N 5.2	68.3 6.5 4.8
II*a*	1	1	57	114–116°[c]	$C_{28}H_{30}N_2O_5S \cdot \frac{1}{2}H_2O$	C 65.2 H 6.0 N 5.4	65.3 6.0 5.4
II*b*	2	1	73	97–99°[c]	$C_{29}H_{32}N_2O_5S \cdot \frac{1}{2}H_2O$	C 65.8 H 6.0 N 5.3	65.7 6.1 5.4
II*c*	3	1	23	105–107°[b]	$C_{30}H_{34}N_2O_5S$	C 67.4 H 6.4 N 5.2	67.5 6.4 5.2
II*d*	1	2	47	132–133°[b]	$C_{29}H_{32}N_2O_5S \cdot \frac{1}{2}H_2O$	C 65.8 H 6.2 N 5.3	65.6 6.2 5.3
II*e*	2	2	42	115–117°[c]	$C_{30}H_{34}N_2O_5S \cdot \frac{1}{2}H_2O$	C 66.3 H 6.3 N 5.2	66.1 6.4 5.0
II*f*	3	2	27	79–82°[b]	$C_{31}H_{36}N_2O_5S \cdot \frac{1}{2}H_2O$	C 66.8 H 6.4 N 5.0	66.9 6.4 5.3

[a] The compounds were pale-yellow oils and were characterized as their solid *S*-benzylthiuronium salts (1). IR spectra (chloroform) and NMR spectra (deuterochloroform) were consistent with the assigned structures. [b] White solid. [c] Beige solid.

RESULTS AND DISCUSSION

The alkenyl acid esters (III*a*–III*f*) and the epoxy acid esters (II*a*–II*f*) were initially assayed using the procedure previously described (2) for determining potential reversible inhibitors of rat liver β-hydroxy-β-methylglutaryl coenzyme A reductase (Table III). Compounds VII*a* and VII*b* were included so that a comparison could be made between the alkenyl and epoxy acid esters and the corresponding inhibitors with only a hydrocarbon (*n*-butyl) side chain. The assay results shown in Table III indicate that these compounds (II*a*–II*f* and III*a*–III*f*) are indeed inhibitors with activity essentially comparable to VII*a* and VII*b*. No significant loss of activity was noted when either a terminal olefin or epoxide function was introduced into the side chain. The lack of increased activity of III*a*–III*f*, which contain the terminal olefin group, seems to indicate that no charge–transfer site has been located on the enzyme surface.

The epoxy acid esters (II*a*–II*f*) were then examined to determine whether the inhibition noted in Table III was reversible or irreversible. The epoxy acid ester II*d* was not evaluated for irreversible activity due to solubility problems. The epoxy acid esters were evaluated as irreversible inhibitors by preincubating the enzyme assay mixture with the inhibitor prior to the addition of substrate. The exposure of the inhibitors to the enzyme was limited to a rela-

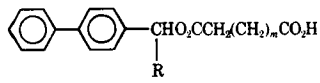
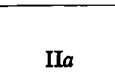
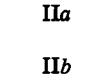


Table III—Inhibition of β-Hydroxy-β-methylglutaryl Coenzyme A Reductase

Compound	R	Activity[a], $(I/S)_{50\%}$, *m* = 1	Compound	Activity[a], $(I/S)_{50\%}$, *m* = 2
II*a*		20	II*d*	13[b] (26%)
II*b*		13	II*e*	13
II*c*		13	II*f*	10
III*a*	$CH_2CH{=}CH_2$	10	III*d*	9
III*b*	$(CH_2)_2CH{=}CH_2$	11	III*e*	11
III*c*	$(CH_2)_3CH{=}CH_2$	11	III*f*	8
VII*a*[c]	$(CH_2)_3CH_3$	11	VII*b*[d]	11

[a] The inhibition index, $(I/S)_{50\%}$, equals the ratio of the micromolar concentration of the inhibitor to the micromolar concentration of the substrate required to give 50% inhibition. At least two sets of duplicate determinations were used. [b] Insoluble at higher concentrations. [c] A standard inhibitor used routinely to provide comparability of each assay set (2). [d] Described previously (2).

tively short time (20 min) because of the instability of the enzyme to these conditions even in the absence of the inhibitors.

Two substrate concentrations (31 and 172 μM) were used in an attempt to detect any irreversible inhibition. In experiments utilizing 31 μM, the same degree of inhibition was observed as that previously found without prior exposure of the enzyme to the inhibitor (Table III). In experiments utilizing 172 μM, no inhibition was found. This substrate concentration was sufficient to eliminate all of the reversible inhibition exhibited by VIIa. Thus, it appears that the epoxy acid esters are inactive as irreversible inhibitors of the reductase.

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Continuous-Flow System for Determination of Diffusion Coefficients: Use of a Natural Membrane

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Abstract □ A natural membrane was employed in an automated diffusion system. A mature male Mongolian gerbil sebaceous gland pad was excised and mounted into a suitable retainer so that the external surface was oriented toward the concentrated aqueous drug solution. Aqueous solutions of benzoic acid and the three commonly used parabens were studied. The gerbil sebaceous pad effectively prevented any diffusion of these drug solutions within 15 hr. Water by itself, however, was transported through the skin even against a pressure gradient. Although no apparent diffusion of these compounds occurred, a significant amount of drug was retained by the sebaceous pad. An expression for membrane–water partition coefficients could be calculated. Based upon thicknesses of natural and synthetic membranes, theoretical approximations of diffusion rates were found using lag time calculations.

Keyphrases □ Diffusion coefficients—determined in automated continuous-flow system, gerbil sebaceous pad □ Partition coefficients—determined in automated continuous-flow system, gerbil sebaceous pad □ Membranes, natural—gerbil sebaceous pad in automated continuous-flow system, diffusion coefficients

The outer surface of the skin is an unspecific, rugged, impermeable membrane, allowing few molecules to penetrate readily. This nonspecific barrier of the skin slows the transfer of all substances; severe chemical treatment may be required to increase the rate of penetration. When the epidermis is separated from the dermis, the epidermis retains all of the resistance to diffusion through skin.

Skin permeability has been studied for more than 50 years, and an enormous quantity of qualitative data has been generated. However, few reports have appeared on the methods or experiments for routine *in vitro* evaluation of the diffusion process in natural skin membranes.

An improvement in quantifying penetration rates came with the use of excised skin in diffusion cells (1). Diffusion cells employing excised skin are capable of producing rapid and reproducible results. Their use depends upon three assumptions.

1. Dead skin has the same permeability properties as living membranes, a relationship that has been shown to hold for several compounds (1).
2. In the diffusion cell, the penetrant must pass through the full thickness of the dermis; whereas in the natural state, the capillary network between the dermis and epidermis can remove the drug (2).
3. The excised skin surface properties remain constant for a reasonable length of time.

The chief advantage of the diffusion cell over the intact animal is the ease in measurement of the penetrant, which is not diluted or altered by the body (3).

EXPERIMENTAL

Chemicals—The drugs whose diffusion constants were to be