# Hypocholesterolemic Agents II: Inhibition of β-Hydroxy-β-methylglutaryl Coenzyme A Reductase by Arylalkyl Hydrogen Succinates and Glutarates

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**Abstract** ☐ A series of arylalkyl hydrogen succinates and glutarates was synthesized and assayed for inhibition of yeast β-hydroxy-β-methylglutaryl coenzyme A reductase. The following compounds were found to be particularly active as inhibitors: 1-(4-biphenylyl)-pentyl hydrogen succinate (5S), 1-(4-biphenylyl)-hexyl hydrogen succinate (6S), 1-(4-biphenylyl)-pentyl hydrogen glutarate (5G), 1-(2-fluorenyl)-pentyl hydrogen succinate (13S), and 1-(2-fluorenyl)-pentyl hydrogen glutarate (13G). Further studies with Compound 5S indicated that the inhibition was reversible and noncompetitive with respect to β-hydroxy-β-methylglutaryl coenzyme A and NADPH. Dixon and modified Hill plots were used to calculate a cooperativity number of 3 for 5S.

**Keyphrases** ☐ Arylalkyl hydrogen succinates and glutarates—synthesis, inhibition of yeast β-hydroxy-β-methylglutaryl-CoA reductase ☐ β-Hydroxy-β-methylglutaryl-CoA reductase—inhibition by arylalkyl hydrogen succinates and glutarates ☐ Inhibition of yeast β-hydroxy-β-methylglutaryl-CoA reductase—synthesis and evaluation of arylalkyl hydrogen succinates and glutarates ☐ Hypocholesterolemic agents—inhibition of β-hydroxy-β-methylglutaryl-CoA reductase by arylalkyl hydrogen succinates and glutarates

Atherosclerosis and its complications constitute a growing problem in public health. Although the term atherosclerosis is ill defined (1), it is generally agreed to include lipid accumulation within the arterial intima as one of its early causative factors.

The discovery of cholesterol in atherosclerotic arteries (2) coupled with the observation of experimentally induced atherosclerosis after cholesterol feeding (3) provided some of the first indications of the implication of cholesterol in atherosclerosis and a suggestion of its causative role.

Because of the importance attached to the plasma cholesterol level in atherosclerosis, many approaches (4, 5) have been investigated to control it. These methods may be summarized as: (*a*) decreased ingestion of cholesterol, (*b*) decreased absorption of cholesterol,

$$R_1-\underset{\substack{|\\R_2}}{CH}-O-\overset{\substack{O\\\|}}{C}-CH_2(CH_2)_n-CO_2H$$

I: $n$ = 1 or 2

(*c*) increased conversion of cholesterol to bile acids, and (*d*) decreased synthesis of cholesterol.

The inhibition of the endogenous cholesterol synthesis could lead to a lowering of the serum level. A brief outline of the biosynthetic pathway is shown in Scheme I.

The specific step of the biosynthetic pathway to be inhibited or blocked is an important factor (6). Inhibition of the first portion of the pathway (acetyl-CoA → acetoacetyl-CoA) is not desirable because acetoacetyl-coenzyme A (CoA) is involved in fatty acid biosynthesis. Antimetabolites related to acetoacetyl-CoA would possibly tend to inhibit fatty acid biosynthesis as well as β-hydroxy-β-methylglutaryl-CoA synthesis; thus the earliest feasible step for control would appear to be the conversion of β-hydroxy-β-methylglutaryl-CoA to mevalonic acid. Later possible sites of inhibition would involve the reaction sequence between mevalonic acid and squalene. However, it appears that any site after mevalonic acid requires almost a complete enzyme inhibition to affect significantly overall cholesterol biosynthesis (7). If the pathway is blocked after squalene synthesis, large amounts of the intermediate accumulate since the reactions after β-hydroxy-β-methylglutaryl-CoA synthesis are irreversible. This accumulation has led to serious toxic effects, as in the case of triparanol (6).

Thus, the conversion of β-hydroxy-β-methylglutaryl-CoA to mevalonic acid seems to be the ideal

acetyl-CoA
↓ ↑
acetoacetyl-CoA
↓ ↑
β-hydroxy-β-methylglutaryl-CoA
↓
mevalonic acid
↓
squalene
↓
lanosterol
↓
desmosterol
↓
cholesterol

*Scheme I*

$$R_1-\overset{\substack{O\\\|}}{C}H + R_2MgX \longrightarrow$$

$$R_1-\underset{\substack{|\\R_2}}{CH}-OH + \begin{matrix} CH_2-C(=O) \\ | \quad\quad O \\ (CH_2)_n-C(=O) \end{matrix} \xrightarrow{\text{pyridine}}$$

$n$ = 1 or 2

$$R_1-\underset{\substack{|\\R_2}}{CH}-O-\overset{\substack{O\\\|}}{C}-CH_2(CH_2)_n-CO_2H$$

I: $n$ = 1 or 2

*Scheme II*

**Table I—Properties of Alkylarylcarbinols**

$R_1—CHOH$
$|$
$R_2$

| Compound Number | $R_1$ | $R_2$ | Yield, % | Meling Point or Boiling Point/mm. | Formula | Analysis, % Calc. | Analysis, % Found |
|---|---|---|---|---|---|---|---|
| 1 | | H— | — | 99–101°[a] | — | — | |
| 2 | | $CH_3$— | 86 | 96–98°[b] | — | — | |
| 3 | | $CH_3CH_2$— | 63 | 57–59°[c] | — | — | |
| 4 | | $CH_3(CH_2)_2$— | 58 | 59–61° | $C_{16}H_{18}O$ | C 84.9<br>H 8.0 | 85.1<br>8.3 |
| 5 | | $CH_3(CH_2)_3$— | 89 | 78–79° | $C_{17}H_{20}O$ | C 85.0<br>H 8.3 | 85.1<br>8.4 |
| 6 | | $CH_3(CH_2)_4$— | 69 | 81–82° | $C_{18}H_{22}O$ | C 85.0<br>H 8.7 | 85.3<br>9.1 |
| 7 | | $CH_3(CH_2)_5$— | 73 | 61–63° | $C_{19}H_{24}O$ | C 85.1<br>H 9.0 | 84.7<br>9.1 |
| 8 | | $CH_3(CH_2)_3$— | — | 100–104°/3[a] | — | — | |
| 9 | Cl— | $CH_3(CH_2)_3$— | 67 | 110°/1[d] | — | — | |
| 10 | Cl, Cl— | $CH_3(CH_2)_3$— | 94 | 135°/0.2 | $C_{11}H_{14}Cl_2O$ | C 56.7<br>H 6.0 | 56.5<br>6.0 |
| 11 | | $CH_3(CH_2)_3$— | 69 | 63–65°[e] | — | — | |
| 12 | | $CH_3(CH_2)_3$— | 58 | 49–50° | $C_{15}H_{18}O$ | C 84.0<br>H 8.4 | 83.8<br>8.3 |
| 13 | | $CH_3(CH_2)_3$— | 90 | 102–102.5° | $C_{18}H_{20}O$ | C 85.7<br>H 7.9 | 85.6<br>8.3 |
| 14 | $C_6H_5CH_2O$ | $CH_3(CH_2)_3$— | 73 | 189°/0.3 | $C_{18}H_{22}O_2$ | C 80.0<br>H 8.1 | 79.8<br>7.9 |
| 15 | $C_6H_5CH_2O$— | $CH_3(CH_2)_3$— | 92 | 67–69.5° | $C_{18}H_{22}O_2$ | C 80.0<br>H 8.1 | 80.1<br>8.2 |

[a] Sample obtained from Aldrich Chemical Co. [b] Lit. (20) m.p. 98–99°. [c] Lit. (13) m.p. 58°. [d] Lit. (13) b.p. 164°/27. [e] Lit. (21) m.p. 65–66°.

point for the inhibition of cholesterol biosynthesis. The selection of this step is based on the following reasons:

1. All reactions prior to mevalonic acid synthesis are either reversible directly or reversible through other enzymatic pathways. Therefore, the problem of accumulation of intermediates is eliminated.

2. This conversion is the rate-limiting step in the synthesis of cholesterol.

3. The physiological regulation of hepatic cholesterogenesis appears to be the result of a feedback inhibition of this step (8, 9) by cholesterol or a lipoprotein containing cholesterol by decreasing the amount, rather than simply the activity, of β-hydroxy-β-methylglutaryl-CoA reductase (10).

The enzyme β-hydroxy-β-methylglutaryl-CoA reductase (E.C.1.1.1.34), which catalyzes this step, is readily available from yeast (11, 12). This enzymatic reduction can be measured spectrophotometrically and thereby provides an *in vitro* assay.

Palazzo *et al.* (13) and Bizzi *et al.* (14) reported that arylalkyl hydrogen succinates and glutarates (I) exhibited hypocholesterolemic activity in rats and also markedly inhibited the *in vitro* incorporation of acetate and mevalonate into both cholesterol and fatty acids. These data suggest that multiple sites of inhibition are involved. The similarity of β-hydroxy-β-methylglutaryl-CoA to the inhibitors (I) in terms of a free carboxyl group, an esterified carboxyl group, and the distance separating these two functional groups suggests that one site of inhibition may be the conversion of β-hydroxy-β-methylglutaryl-CoA to mevalonic acid. Therefore, a series of arylalkyl hydrogen succinates and

Table II—Properties of Succinates and Glutarates

$R_1—CH(R_2)O_2CCH_2(CH_2)_nCO_2H$

| Compound Number | $R_1$ | $R_2$ | Yield, % | Melting Point | Melting Point (Salt) | Formula | Analysis, %[a] Calc. | Analysis, %[a] Found |
|---|---|---|---|---|---|---|---|---|
| | | | | **Succinates, $n = 1$** | | | | |
| 1S | | H— | 98 | 135–138° | — | $C_{17}H_{16}O_4$ | C 71.8<br>H 5.6 | 71.7<br>5.6 |
| 2S | | $CH_3$— | 60 | 109–111° | — | $C_{18}H_{18}O_4$ | C 72.5<br>H 6.0 | 72.7<br>6.4 |
| 3S | | $CH_3CH_2$— | 27 | 64–66°[b] | — | — | — | — |
| 4S | | $CH_3(CH_2)_2$— | 45 | 73–74° | — | $C_{20}H_{22}O_4$ | C 73.6<br>H 6.6 | 73.5<br>6.9 |
| 5S | | $CH_3(CH_2)_3$— | 68 | 77–79°[c] | — | — | — | — |
| 6S | | $CH_3(CH_2)_4$— | 60 | 59–61° | — | $C_{22}H_{26}O_4$ | C 74.5<br>H 7.4 | 74.6<br>7.7 |
| 7S | | $CH_3(CH_2)_5$— | 66 | 49–52° | — | $C_{23}H_{28}O_4$ | C 75.0<br>H 7.6 | 75.4<br>7.9 |
| 8S | | $CH_3(CH_2)_3$— | 67 | Oil | 127–128° | $C_{23}H_{30}N_2O_4S$ | C 64.2<br>H 7.0<br>N 6.5 | 64.0<br>7.0<br>6.3 |
| 9S | Cl | $CH_3(CH_2)_3$— | 76 | Oil | — | $C_{15}H_{19}ClO_4$ | C 60.7<br>H 6.4 | 60.6<br>6.5 |
| 10S | Cl, Cl | $CH_3(CH_2)_3$— | 33 | Oil | 123–123.5° | $C_{23}H_{28}Cl_2N_2O_4S$ | C 55.3<br>H 5.6<br>N 5.6 | 55.2<br>5.6<br>5.5 |
| 11S | | $CH_3(CH_2)_3$— | 67 | Oil | 118–118.5° | $C_{27}H_{32}N_2O_4S$ | C 67.5<br>H 6.7<br>N 5.6 | 67.4<br>6.8<br>5.8 |
| 12S | | $CH_3(CH_2)_3$— | 55 | 72–72.5° | — | $C_{19}H_{22}O_4$ | C 72.7<br>H 7.0 | 72.8<br>7.0 |
| 13S | | $CH_3(CH_2)_3$— | 53 | 104–106° | — | $C_{22}H_{24}O_4$ | C 75.0<br>H 6.8 | 75.0<br>7.1 |
| 14S | $C_6H_5CH_2O$ | $CH_3(CH_2)_3$— | 37 | 78–80° | — | $C_{22}H_{26}O_5$ | C 71.4<br>H 7.0 | 71.4<br>7.2 |
| 15S | $C_6H_5CH_2O$— | $CH_3(CH_2)_3$— | 25 | 75.5–77° | — | $C_{22}H_{26}O_5$ | C 71.4<br>H 7.0 | 71.3<br>7.3 |
| | | | | **Glutarates, $n = 2$** | | | | |
| 1G | | H— | 80 | 90–91° | — | $C_{18}H_{18}O_4$ | C 72.5<br>H 6.1 | 72.3<br>6.1 |
| 2G | | $CH_3$— | 40 | 58–61° | — | $C_{19}H_{20}O_4$ | C 73.1<br>H 6.4 | 73.1<br>6.7 |
| 3G | | $CH_3CH_2$— | 40 | 52–55°[d] | 144–145° | $C_{28}H_{32}N_2O_4S$ | C 68.3<br>H 6.5<br>N 5.7 | 68.4<br>6.5<br>5.8 |
| 4G | | $CH_3(CH_2)_2$— | 31 | Oil | 141–142° | $C_{29}H_{34}N_2O_4S$ | C 68.7<br>H 6.7<br>N 5.5 | 68.5<br>6.9<br>5.6 |
| 5G | | $CH_3(CH_2)_3$— | 53 | Oil | 122–123° | $C_{30}H_{36}N_2O_4S$ | C 69.2<br>H 6.9<br>N 5.4 | 69.4<br>7.1<br>5.3 |
| 6G | | $CH_3(CH_2)_4$— | 40 | Oil | 106–107° | $C_{31}H_{38}N_2O_4S$ | C 69.7<br>H 7.1<br>N 5.3 | 70.0<br>7.1<br>5.5 |
| 7G | | $CH_3(CH_2)_5$— | 43 | Oil | 105–107° | $C_{32}H_{40}N_2O_4S$ | C 70.1<br>H 7.3<br>N 5.1 | 69.9<br>7.0<br>5.0 |
| 8G | | $CH_3(CH_2)_3$— | 52 | Oil[e] | — | — | — | — |
| 9G | Cl | $CH_3(CH_2)_3$— | 48 | Oil | — | $C_{16}H_{21}ClO_4$ | C 61.4<br>H 6.7 | 61.7<br>6.6 |

**Table II**—(*Continued*)

| Compound Number | $R_1$ | $R_2$ | Yield, % | Melting Point | Melting Point (Salt) | Formula | Analysis, %[a] Calc. | Found |
|---|---|---|---|---|---|---|---|---|
| | | | | **Glutarates, *n* = 2** | | | | |
| 10G | Cl, Cl | $CH_3(CH_2)_3$— | 29 | Oil | 118–120° | $C_{24}H_{30}Cl_2N_2O_4S$ | C 56.1<br>H 5.9<br>N 5.5 | 56.2<br>6.1<br>5.7 |
| 11G | | $CH_3(CH_2)_3$— | 48 | Oil | 112–114° | $C_{28}H_{34}N_2O_4S$ | C 68.0<br>H 6.9<br>N 5.7 | 68.2<br>7.1<br>5.7 |
| 12G | | $CH_3(CH_2)_3$— | 53 | Oil | 116–117° | $C_{28}H_{34}N_2O_4S$ | C 68.0<br>H 6.9<br>N 5.7 | 68.2<br>7.0<br>5.5 |
| 13G | | $CH_3(CH_2)_3$— | 40 | Oil | 129–130° | $C_{31}H_{36}N_2O_4S$ | C 70.0<br>H 6.8<br>N 5.3 | 69.8<br>6.8<br>5.1 |
| 14G | $C_6H_5CH_2O$ | $CH_3(CH_2)_3$— | 21 | Oil | 116.5–118.5° | $C_{31}H_{38}N_2O_5S$ | C 67.3<br>H 6.9<br>N 5.1 | 67.4<br>7.0<br>4.9 |
| 15G | $C_6H_5CH_2O$— | $CH_3(CH_2)_3$— | 40 | Oil | 110–112° | $C_{31}H_{38}N_2O_5S$ | C 67.3<br>H 6.9<br>N 5.1 | 67.2<br>7.1<br>5.0 |

[a] Analytical data were obtained on the solid monoesters, while the oils were characterized as their solid S-benzylthiuronium salts. Th) formula indicates the compound upon which analytical data were obtained. [b] Lit. (13) m.p. 66°. [c] Lit. (13) m.p. 80°. [d] Lit. (13) 62°. [e] Lit. (13e $n_D^{20}$ 1.4961. Found: $n_D^{20}$ 1.4957.

glutarates (I) were prepared and assayed as inhibitors of the reductase.

The synthetic scheme for the inhibitors is shown in Scheme II. Treatment of the appropriate Grignard reagent with an ethereal solution of a suitable aldehyde gave the desired alcohols (Table I). Acylation of the alcohols with succinic or glutaric anhydride in dry pyridine afforded the desired monoesters (Table II).

The monoesters[1] that failed to crystallize were chromatographed on silicic acid and characterized as their solid S-benzylthiuronium salts.

## EXPERIMENTAL[2]

**Preparation of Alkylarylcarbinols**—The carbinols listed in Table I were prepared by treatment of the appropriate aromatic aldehyde with the required Grignard reagent. The preparation of 1-(4-biphenylyl)pentanol may serve as an example.

**1-(4-Biphenylyl)pentanol (Compound 5)**—The Grignard reagent was prepared from 4.1 g. (0.17 g.-atom) of magnesium turnings and 20 ml. (26 g., 0.192 mole) of *n*-butyl bromide in 100 ml. of ether. To the prepared Grignard was added dropwise, without heating or cooling, 25 g. (0.137 mole) of 4-biphenylcarboxaldehyde in 200 ml. of ether. The mixture was stirred at 25° for 45 min. after the addition was complete. A solution of saturated ammonium chloride, adjusted to pH 7 with ammonium hydroxide, was added dropwise with cooling until a heavy granular precipitate settled out. The mixture was filtered and the solvent was removed under reduced pressure to afford 29.5 g., 89%, of a white solid, m.p. 77–78°. Recrystallization from ethyl acetate–petroleum ether, b.p. 60–75°, afforded an analytical specimen, m.p. 78–79°. Table I lists the analytical data.

**Preparation of Arylalkyl Hydrogen Succinates and Glutarates**—The succinates and glutarates listed in Table II were prepared using a modification of the pyridine method described by Palazzo *et al.* (13). The preparation of 1-(4-biphenylyl)-pentyl hydrogen glutarate may serve as an example.

**1-(4-Biphenylyl)-pentyl Hydrogen Glutarate (Compound 5G)**—A mixture of 3.6 g. (0.015 mole) of the alcohol (Compound 5), 1.71 g. (0.015 mole) of glutaric anhydride, and 20 ml. of pyridine (distilled from and stored over barium oxide) was heated at reflux for 8 hr. The resulting solution was poured on an ice–hydrochloric acid mixture and then extracted with ether. The organic phase was washed twice with water and then twice with a 5% sodium hydroxide solution. The basic aqueous extract was acidified with a 5% hydrochloric acid solution and then was extracted with ether. The organic phase was washed with water, washed with a saturated sodium chloride solution, and then dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure to give 2.9 g. of a colorless oil. The colorless oil was chromatographed on 50 g. of silicic acid. Elution with 20% ether–petroleum ether, b.p. 60–75°, afforded 2.5 g. of a colorless oil (Compound 5G), 46%. The NMR spectrum ($CDCl_3$) exhibited a 9:1 ratio of aromatic protons to the carboxyl proton, indicating the desired monoester. Analytical data were obtained for the solid monoesters, while the oily monoesters were characterized as their solid S-benzylthiuronium salts (Table II).

**Preparation of S-Benzylthiuronium Salts**—The method of Donleavy (15) was modified. To a solution of 0.17 g. (0.49 mmole) of 5G in 15 ml. of 95% ethanol was added 5.1 ml. of 0.095 *M* sodium hydroxide solution. To this solution was added 0.5 g. (2.4 mmoles) of 2-benzyl-2-thiopseudourea hydrochloride[3] in 5 ml. of hot 95% ethanol and then 20 ml. of water. The salt precipitated upon cooling to give 0.21 g. of a white solid. Two recrystallizations from ethyl acetate–petroleum ether, b.p. 60–75°, afforded an analytical specimen as white prisms, m.p. 122–123°. Table II lists the analytical data.

**Enzyme Preparation**—For the comparative inhibitor studies, β-hydroxy-β-methylglutaryl-CoA reductase was prepared from locally obtained baker's yeast[4] by the procedure of Durr and Rudney (11) as previously reported (12). The aqueous enzyme preparation was transferred to capped serum bottles and stored at −10° until used.

[1] The monoester 9S was characterized as an oil.

[2] Melting points, determined with a Thomas-Hoover capillary melting-point apparatus, are uncorrected. Microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. NMR spectra were obtained using a Varian A-60 NMR spectrophotometer.

[3] Eastman.

[4] Fleischman's.

**Table III**—Inhibition of β-Hydroxy-β-methylglutaryl-CoA Reductase

| Compound Number | Activity $I/S$[a] | Activity Percent Inhibition | Compound Number | Activity $I/S$[a] | Activity Percent Inhibition |
|---|---|---|---|---|---|
| 1S | 150 | 0 | 1G | 150 | 0 |
| 2S | Insoluble | — | 2G | Insoluble | — |
| 3S | 150 | 16 | 3G | 150 | 50 |
| 4S | 150 | 44 | 4G | 90 | 50 |
| 5S | 66 | 50 | 5G | 62 | 50 |
| 6S | 62 | 50 | 6G | 75 | 50 |
| 7S | Insoluble | — | 7G | 50 | 30 |
| 8S | 100 | 0 | 8G | 100 | 0 |
| 9S | 100 | 0 | 9G | 100 | 0 |
| 10S | 100 | 30 | 10G | 100 | 50 |
| 11S | 150 | 50 | 11G | 125 | 50 |
| 12S | 115 | 50 | 12G | 95 | 50 |
| 13S | 70 | 50 | 13G | 65 | 50 |
| 14S | 125 | 50 | 14G | 125 | 50 |
| 15S | 115 | 50 | 15G | 130 | 50 |

[a] The inhibition index, $I/S$, equals the ratio of the n$M$ concentration of the inhibitor to the n$M$ concentration of substrate. The $I/S$ ratios are reported for 50% inhibition unless the solubility properties of the inhibitor precludes this determination. If sufficiently soluble, a maximum ratio of 150 was used for compounds exhibiting a low order of inhibition. The $I/S_{50\%}$ ratios were determined by interpolation from a plot of percent inhibition *versus* log $I/S$ ratio. At least three sets of duplicate determinations between 30 and 80% inhibition were used, and one set within 45–55% inhibition was used for confirmation.

**Enzyme Inhibition**—Enzyme activity was determined at 30° in a recording spectrophotometer[5] by measuring the decrease in absorbance at 340 nm. for the oxidation of NADPH (12). To each cell were added 1.6 ml. of 0.15 $M$ $NaH_2PO_4$ (pH 7.0) containing 1 m$M$ ethylenediaminetetraacetate, 0.2 ml. ethylene glycol monoethyl ether containing inhibitor, 0.5 ml. of a solution containing 6.8 mg. cysteine, 50 μl. reduced triphosphopyridine nucleotide (NADPH) solution containing 0.36 μmole NADPH, and 0.1 ml. enzyme solution (about 14 mg. protein/ml. in buffer). In control cells, ethylene glycol monoethyl ether was added without inhibitor. The absorbance was allowed to stabilize after stirring prior to adding 50 μl. of β-hydroxy-β-methylglutaryl-CoA solution containing 44 nmoles of *dl*-β-hydroxy-β-methylglutaryl-CoA (12) and stirring again.

**Kinetic Studies**—For the kinetic studies, a more highly purified enzyme was prepared from baker's yeast by a modification of the methods of Durr and Rudney (11) and Kirtley and Rudney (16) as reported previously (17). This procedure adds a 30–45% saturated ammonium sulfate precipitation and substitutes 1 m$M$ dithiothreitol for cysteine.

Reversibility of inhibition by dialysis was also investigated. Various combinations of 5S, substrate, coenzyme, and enzyme were dialyzed for 6, 12, or 24 hr. against buffer; activity was compared to enzyme alone subjected to similar dialysis (Table IV).

## RESULTS AND DISCUSSION

Thirty monoesters of succinic and glutaric acids were prepared and assayed as inhibitors of yeast β-hydroxy-β-methylglutaryl-CoA reductase (Table III). The compounds are composed of two series, the succinates (S) and the glutarates (G). This study involved the modification of the alcohol moiety of the ester function to determine what structural aspects were essential for activity. The complex alcohol moiety is composed of aromatic ($R_1$) and alkyl ($R_2$) groups (Structure I). To differentiate between the effects of the aromatic and alkyl groups, Compounds 1S–7S and 1G–7G were prepared and assayed. The aromatic group ($R_1$) was maintained as a biphenyl group with the alkyl group ($R_2$) being varied from hydrogen to hexyl. As the chain length is increased from $R_2$ = hydrogen to $R_2$ = *n*-hexyl, activity increases dramatically, with a maximum resulting when $R_2$ = *n*-butyl or *n*-pentyl (5S, 5G, 6G). In the glutarate series, the ethyl compound, 3G, begins to show

[5] Gilford.

**Table IV**—Dialysis of Enzyme in the Presence of Substrate, Coenzyme, and Inhibitor 5S[a]

| | Hours of Dialysis: Experiment I, 6 | Experiment I, 12 | Experiment II, 12 | Experiment II, 24 |
|---|---|---|---|---|
| Control | 100.0% | 100.0% | 100.0% | 100.0% |
| Inhibitor (1.58 m$M$) | 79.4 | 83.3 | 97.2 | 100.5 |
| Inhibitor (1.58 m$M$), β-hydroxy-β-methylglutaryl-CoA (15.8 μ$M$) | 22.4 | 42.8 | 47.6 | 91.1 |
| Inhibitor (1.58 m$M$), NADPH (144 μ$M$) | 80.9 | 93.3 | 104.7 | 94.2 |
| Inhibitor (1.58 m$M$), β-hydroxy-β-methylglutaryl-CoA (15.8 μ$M$), NADPH (144 μ$M$) | 34.0 | 51.7 | 68.8 | 102.1 |

[a] The indicated enzyme solutions containing 14.8 mg. enzyme were diluted to 6.0 ml. with phosphate buffer (pH 7.6) containing 1 m$M$ dithiothreitol, mixed, incubated for 5 min. at 30°, and transferred to dialysis tubing. After dialysis at 5° against phosphate buffer (pH 7.6) containing 1 m$M$ dithiothreitol for 6, 12, or 24 hr., enzyme activity was determined as before and calculated as a percentage of control activity (dialyzed without inhibitor, β-hydroxy-β-methylglutaryl-CoA, or NADPH).

reasonable activity ($I/S_{0.5}$ = 150), whereas the ethyl compound in the succinate series, 3S, indicates only minimal activity ($I/S$ = 150, 16% inhibition). However, when $R_2$ = *n*-butyl in both series, 5S and 5G, the activities become equal. An additional methylene group, Compound 6S, fails to increase activity in the succinate series; in the glutarate series, Compound 6G, a slight decrease in activity results. A further increase of chain length of $R_2$ is precluded by solubility problems with Compounds 7S and 7G. Thus, it appears that a hydrophobic bonding area on the enzyme has such dimensions that it can accommodate a chain length up to

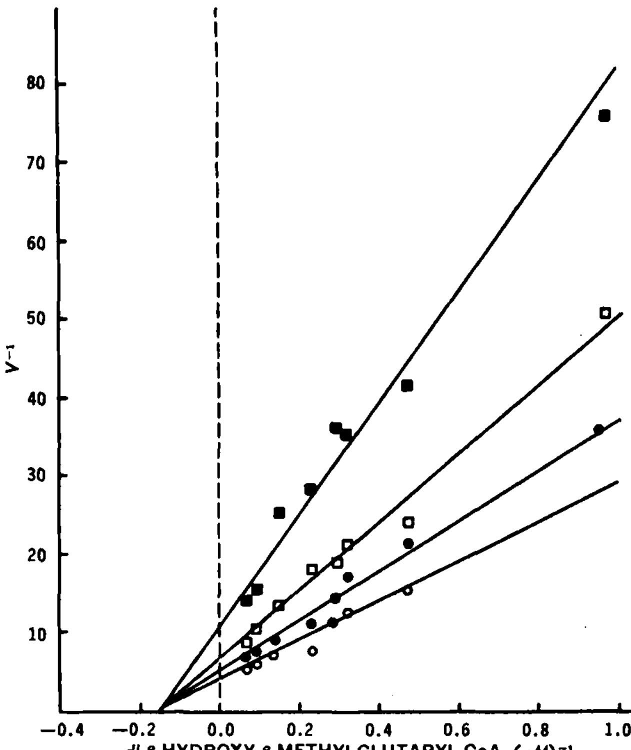


**Figure 1**—*Double-reciprocal plot of velocity* ($\Delta A_{340}$/*min.*) *versus substrate concentration. All reaction mixtures consist of 7.4 mg. enzyme, 0.1 ml. ethylene glycol monoethyl ether, 2.4 ml. of 0.15* M *phosphate buffer containing 1* mM *dithiothreitol (pH 7.6), 0.36 μmole of NADPH, and varying amounts of substrate in a total volume of 2.5 ml. Key:* ○, *uninhibited;* ●, *0.79* mM *5S;* □, *1.19* mM *5S; and* ■, *1.58* mM *5S.*

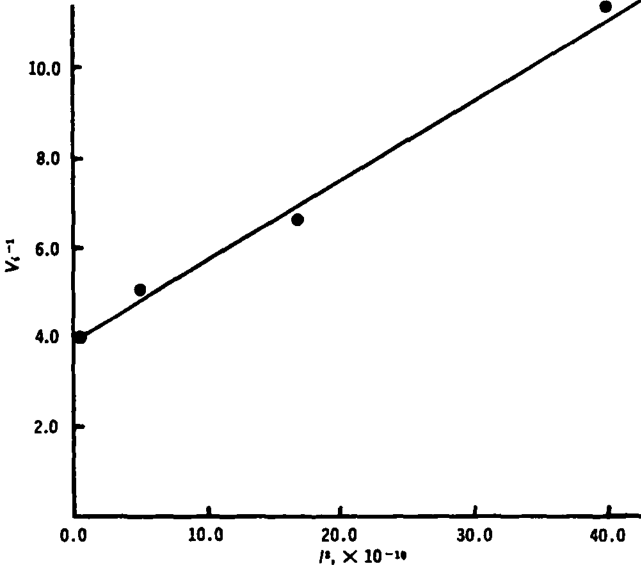


**Figure 2**—*Dixon plot for inhibition by 5S (data from Fig. 1).*

five carbon atoms.

The effects of the variation of the aromatic group ($R_1$) was investigated to determine if hydrophobic and electronic effects are important in binding of the inhibitor to the enzyme. The *n*-butyl chain ($R_2$) was retained in these inhibitors. The activity of Compounds 5S, 5G, 8S–10S, and 8G–10G appears to indicate that hydrophobic bonding is of primary importance in determining the degree of binding. As the hydrophobic bonding characteristics of the group ($R_1$) is increased (biphenyl > 3,4-dichlorophenyl > 4-chlorophenyl > phenyl), the activity of the monoesters increases. Coplanarity of the biphenyl group also appears to be important, as shown by the similar activity of the planar fluorenyl group, Compounds 13S and 13G, when compared to the biphenyl group, Compounds 5S and 5G.

In an attempt to determine if the position of attachment of the hydrophobic group ($R_1$) to the carbon atom bearing the ether oxygen of the ester was critical, the isomeric $\alpha$- and $\beta$-naphthyl derivatives (11S, 11G, 12S, and 12G) were examined. No appreciable differences in activity were observed. An attempt also was made to detect electronic effects by evaluating positional isomers (14S, 14G, 15S, and 15G) containing an electron-donating substituent. This variation produced no significant change in activity.

In an effort to obtain more detailed information concerning the inhibition, Compound 5S was selected for additional studies on the type of inhibition, cooperativity, and reversal of inhibition by dialysis. From a double-reciprocal plot of velocity *versus* $\beta$-hydroxy-$\beta$-methylglutaryl-CoA (Fig. 1), it may be seen that the inhibition is noncompetitive with respect to substrate. A similar double-reciprocal plot of velocity *versus* NADPH (not shown) indicated that the inhibition is noncompetitive with respect to coenzyme as well.

A Dixon plot (18) and a modified Hill plot (19) were used to investigate cooperativity. A cooperativity number of 3 was calculated from the Dixon plot (Fig. 2) as well as a modified Hill plot (not shown). This is subject to a number of alternative explanations:

1. The enzyme molecules must combine with three molecules of inhibitor before any inhibition occurs.

2. The binding of one molecule of inhibitor to an enzyme molecule facilitates further binding of inhibitor to a total of three.

3. The molecules of enzyme which bind fewer than three molecules of inhibitor are quite unstable, either losing all of their bound inhibitor or binding up to their total of three.

Although subunits of this enzyme have not been reported, these data are compatible with an enzyme model consisting of subunits. However, subunits and their interactions are only one explanation of cooperativity.

It may be seen from Table IV that inhibition of the enzyme with inhibitor 5S is reversible by 24 hr. of dialysis. In the absence of substrate, however, inhibition is reversed in 12 hr. This would suggest the formation of a more stable ternary complex of enzyme, inhibitor, and substrate. The presence of NADPH appears to have little effect on the reversal of inhibition.

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