Hypocholesterolemic Agents I: 3-Methyl-4-phenylbutenoic Acids

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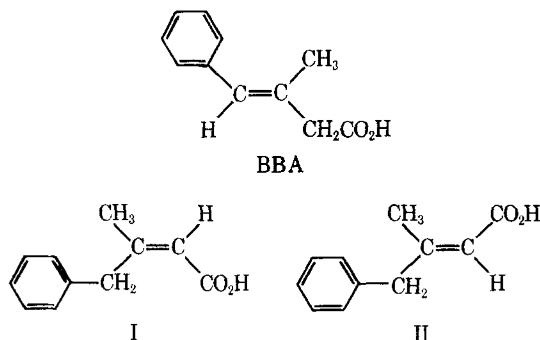
Abstract □ Three isomers of 3-methyl-4-phenylbutenoic acid were examined as potential inhibitors of yeast β-hydroxy-β-methylglutaryl-CoA reductase. All were shown to be inactive when assayed over a concentration range of 8–17 m*M*.

Keyphrases □ 3-Methyl-4-phenylbutenoic acid isomers—tested as β-hydroxy-β-methylglutaryl-CoA reductase inhibitors □ β-Hydroxy-β-methylglutaryl-CoA reductase inhibitors—3-methyl-4-phenylbutenoic acid isomers tested

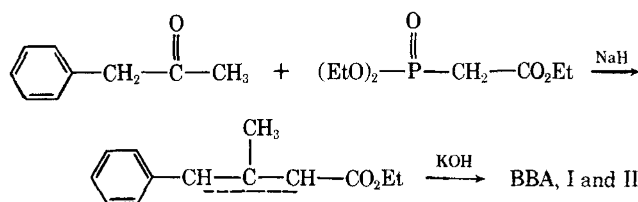
A recent report by Porcellati *et al.* (1) provided evidence for the inhibition of cholesterol biosynthesis (*in vitro*) by β-benzal butyric acid (BBA) at a site located between acetate and mevalonate. Inhibition of acetyl-CoA:ligase (E.C.6.2.1.1) and acetyl-CoA acetyl transferase (E.C.2.3.1) reactions was reported (1) to be absent except at high concentrations.

In an effort to locate the specific site of inhibition, the effects of BBA and its double-bond isomers (I and II) on yeast β-hydroxy-β-methylglutaryl-CoA reductase (HMG-CoA reductase, E.C.1.1.1.34) were investigated.



The synthetic scheme for the butenoic acids is presented in Scheme I.

Treatment of phenylacetone with triethyl phosphonacetate afforded a mixture of the desired α,β- and β,γ-unsaturated esters. Saponification of the mixture and chromatography on a silicic acid column yielded the desired pure acids.



Scheme I

EXPERIMENTAL[1]

Chemical Synthesis—*Preparation of BBA and* cis-(*I*)- *and* trans-(*II*)-*3-Methyl-4-phenyl-2-butenoic Acids*—The procedure of Wadsworth and Emmons (2) was used. To a cooled suspension of 2.4 g. of 50% sodium hydride (0.05 mole) in 100 ml. of dimethoxyethane (distilled from lithium aluminum hydride) was added dropwise with stirring 11.2 g. (0.05 mole) of triethyl phosphonoacetate. The temperature was maintained below 30° during the addition, and the mixture was stirred an additional 45 min. after the addition was complete. To this mixture was added dropwise 6.7 g. (0.05 mole) of phenyl-2-propanone, maintaining the temperature below 30°. The reaction mixture was stirred for 30 min. at 25°, and then 20 ml. of water was added. The mixture was extracted with 300 ml. of ether, 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 a colorless liquid, which was distilled, b.p. 118° (1 mm.), to afford 5.3 g. of a mixture of esters which was not characterized but was saponified directly.

To this mixture of esters were added 3.0 g. of 85% potassium hydroxide pellets, 30 ml. of methanol, and 15 ml. of water. The mixture was heated under reflux for 4 hr.; then the methanol was removed under reduced pressure. The mixture was extracted with ether. The basic aqueous phase was acidified with 5% hydrochloric acid solution and then was extracted with ethyl acetate. 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 2.5 g. of a colorless oil (0.014 mole), 28%.

The colorless oil was chromatographed on 60 g. silicic acid (Table I). Fractions 4 and 5 were combined and rechromatographed on 40 g. of silicic acid (Table II). Fractions 8 and 9 were combined and recrystallized twice from petroleum ether, b.p. 60–75°, to give 130 mg. of BBA as white plates, m.p. 111–112°, lit. (3) m.p. 112–113°.

Fractions 7 and 8 from the second column were combined and recrystallized from petroleum ether to give 51 mg. of I as white prisms, m.p. 66–69°. Table III lists the NMR data.

Anal.—Calcd. for $C_{11}H_{12}O_2$: C, 75.0; H, 6.8. Found: C, 75.2; H, 6.8.

Fractions 10–12 were combined and recrystallized from petroleum ether to give 100 mg. of II as white prisms, m.p. 73–76°. Table III lists the NMR data.

Anal.—Calcd. for $C_{11}H_{12}O_2$: C, 75.0; H, 6.8. Found: C, 74.8; H, 7.0.

Preparation of β-Hydroxy-β-methylglutaric Anhydride[2]—To an ice-cold solution of 2.88 g. (17.8 mmoles) of β-hydroxy-β-methylglutaric acid[3] in 40 ml. of dry acetone was added dropwise with stirring 3.6 g. (17.4 mmoles) of dicyclohexylcarbodiimide[4] in 20 ml. of dry acetone. Immediately, a white precipitate began to form. The mixture was stirred at room temperature for 17 hr. Dry ether (50 ml.) was added, and the mixture was filtered. The solvent was removed under reduced pressure to afford a white solid, which was slurried with ether–petroleum ether. The mixture was cooled and then filtered to give 2.54 g. (17.2 mmoles), 97%, of white prisms, m.p. 100–102° [lit. (5) m.p. 102–103°].

Preparation of HMG-CoA—The method of Louw *et al.* (4) was used, except that the bubbling of nitrogen through the system was found to be unnecessary.

[1] Melting points, determined with a Thomas-Hoover capillary melting-point apparatus, are corrected. Microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn.

[2] The authors experienced the same difficulty as Louw *et al.* (4) in the preparation of β-hydroxy-β-methylglutaric anhydride. Therefore, an alternate method was developed, using dicyclohexylcarbodiimide, which afforded the anhydride in high yield.

[3] Mann Research Lab.

[4] Aldrich Chemical Co.

Table I—Chromatographic Separation of BBA[a]

Fraction	Weight, mg.	Description
1. 100% P.E.	—	
2. 100% P.E.	—	
3. 5% E–P.E.	—	
4. 5% E–P.E.	600	Colorless liquid
5. 5% E–P.E.	500	Colorless liquid
6. 5% E–P.E.	350	Colorless liquid
7. 5% E–P.E.	130	Colorless liquid
8. 50% E–P.E.	400	White solid
9. 100% E	150	White solid
Total	2.130 g.	

[a] Fractions are 100 ml. of petroleum ether (P.E.), b.p. 60–75°; diethyl ether (E); or mixtures as indicated (v/v).

Assay of Substrate—HMG-CoA was assayed by using Ellman's method (6) to determine reduced Coenzyme A before and after saponification (0.05 *N* KOH for 10 min. at 30°). This is similar to the method recently reported by Louw *et al.* (4).

Enzyme Preparation—The enzyme, HMG-CoA reductase, was prepared from locally obtained Fleischman's bakers yeast by the procedure of Durr and Rudney (7). This procedure consists of freezing crumbled yeast in liquid nitrogen, extraction with K_2HPO_4 solution, precipitation at pH 4.7, dissolving the precipitate in 0.15 *M* K_2HPO_4 containing 1.0 m*M* ethylenediamine tetraacetate, titration to pH 7 with 1 *N* KOH, centrifugation at 105,000×*g* for 1 hr. (or 37,000×*g* for 170 min. at 5°), and filtration of the supernatant through glass wool. The enzyme solution was transferred to capped serum bottles and stored at −10° until it was used.

Enzyme Inhibition—Enzyme activity was determined at 30° in a Gilford recording spectrophotometer by measuring the decrease of optical density at 340 nm. according to a modification of the procedure of Durr and Rudney (7). To each cell were added 1.6 ml. of 0.15 *M* NaH_2PO_4 buffer (pH 7.0) containing 1 m*M* ethylenediamine tetraacetate, 0.2 ml. ethylene glycol monoethyl ether, 0.5 ml. aqueous cysteine solution containing 6.8 mg. cysteine, 0.05 ml. reduced triphosphopyridine nucleotide (TPNH) solution containing 0.36 μm. TPNH, and 0.1 ml. enzyme solution (about 14 mg. protein/ml. in buffer). The optical density was allowed to stabilize after stirring prior to the addition of 0.05 ml. HMG-CoA solution containing 44 nmoles of DL-HMG-CoA and stirring again. (Inhibition was studied by dissolving the inhibitor in ethylene glycol monoethyl ether.) Albumin was deleted from the procedure of Durr and Rudney (7) since its addition did not seem to improve the assay.

RESULTS

BBA has been assigned a *cis*-configuration with regard to the phenyl and methyl groups. This assignment was been made on the basis of steric considerations.

Structural assignment of the acids, I and II, was made on the basis of NMR spectroscopy. A tabulation of the NMR data is found in Table III.

Table II—Chromatographic Separation of Compounds I and II[a]

Fraction	Weight, mg.	Description
1. 100% P.E.	—	
2. 100% P.E.	—	
3. 1% E–P.E.	—	
4. 1% E–P.E.	—	
5. 1% E–P.E.	—	
6. 2% E–P.E.	—	
7. 2% E–P.E.	37	White solid
8. 2% E–P.E.	100	White solid
9. 2% E–P.E.	91	Colorless liquid
10. 2% E–P.E.	106	White solid
11. 2% E–P.E.	60	White solid
12. 2% E–P.E.	46	White solid
13. 100% E	544	Yellow oil
Total	984	

[a] Fractions are 100 ml. of petroleum ether (P.E.), b.p. 60–75°; diethyl ether (E); or mixtures as indicated (v/v).

Table III—Chemical Shift Data[a]

Compound	Vinyl	Methylene	Methyl
BBA	6.44	3.24	1.97
I	5.82	4.00	1.77
II	5.73	3.42	2.10
CH_3 (*b*), CH_3 (*c*) C=C H, CO_2H	5.72	—	1.93[b] 2.18[c]

[a] The chemical shifts were determined in deuterochloroform using a Varian A-60 NMR spectrophotometer. The values are reported in p.p.m. relative to tetramethylsilane as an internal standard. All peaks for BBA, I, and II were observed as singlets using a sweep width of 1000 Hz.

The stereochemistry of I and II has been assigned after comparing the chemical shifts of the methyl protons of the acids, I and II, with the methyl protons of 3-methyl-2-butenoic acid.

The methyl protons of I appear at 1.77 p.p.m., which is shifted upfield from the expected position of approximately 1.93 p.p.m. observed when the methyl group is *cis* to the hydrogen atom as in 3-methyl-2-butenoic acid. This diamagnetic shift can be readily explained by the steric crowding of the carboxyl and benzyl groups. To minimize this steric interaction between the two groups, the molecule must assume a conformation that allows shielding of the methyl group by the aromatic ring. Upon examination of Dreiding stereomodels, this conformation would appear to be a favorable one.

The stereochemical assignment of II is also based on the chemical shift of the methyl group. The methyl protons of II appear at 2.10 p.p.m., which is in close agreement with the position of 2.18 p.p.m. observed when the methyl group is *trans* to the hydrogen atom as in 3-methyl-2-butenoic acid.

No inhibition of HMG-CoA reductase activity was noted either for concentrations up to and including 22 μmoles/cell (500 times substrate concentration) of Compounds I and II or for 44 μmoles/cell (1000 times substrate concentration) of BBA.

DISCUSSION

Although it was reported that in rat liver BBA strongly inhibits the *in vitro* incorporation of acetate but not mevalonate into cholesterol (1), the exact step inhibited is not known. Acetyl-CoA: ligase from pigeon liver is 58% inhibited by 10 m*M* BBA, but acetyl-CoA acetyl transferase from pigeon liver shows no inhibition in the presence of 20 m*M* BBA. The finding that even acetyl-CoA:ligase was not inhibited at concentrations of BBA below 1 m*M*, which markedly inhibited incorporation of acetate into sterol, suggested to Porcellati *et al.* (1) that another enzyme between acetate and mevalonate was also inhibited by BBA. The present investigation clearly shows no inhibition of HMG-CoA reductase at concentrations up to 17 m*M* BBA. Even though the degree of similarity between HMG-CoA reductase from yeast and that from mammalian sources is not known, other known inhibitors of mammalian cholesterol biosynthesis also have been observed to be potent inhibitors of yeast HMG-CoA reductase (8).

Having investigated the *in vitro* effect of BBA on yeast HMG-CoA reductase, the authors intend to investigate the effect of mammalian HMG-CoA reductase in a similar manner. The present results suggest, however, that BBA inhibits yet another step between acetate and mevalonate. Alternatively, it could increase the activity of other enzymes that utilize intermediates of the acetate ⟶ mevalonate pathway and thus make these intermediates unavailable. For example, an increase in activity of certain enzymes of fatty acid biosynthesis would be expected to result in less substrate available for mevalonic acid biosynthesis.

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Sterically Hindered Esters of Vitamin A III: Biological Availability of Vitamin A from Sterically Hindered Esters

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Abstract ☐ Previous studies demonstrated that sterically hindered esters of vitamin A, such as the α,α-dimethylpalmitate and α-methyl-α-ethylcaproate, had some superior chemical properties compared to the palmitate. The biological availability of vitamin A from these esters, compared with the palmitate and cod liver oil as controls, was determined in rats. Maximum availability of vitamin A was obtained with the palmitate followed by cod liver oil. Availability from the α,α-dimethylpalmitate was 71% that of the palmitate, while the α-methyl-α-ethylcaproate had a poor availability pattern.

Keyphrases ☐ Vitamin A—biological availability from sterically hindered esters ☐ Biological availability—vitamin A from sterically hindered esters ☐ Biological assay—comparison of vitamin A esters

A previous study by Forlano and Harris (1) suggested that maximal stability of vitamin A esters could be obtained by the introduction of electropositive groups in the α-position of the esters. Based on this concept, sterically hindered esters of vitamin A were prepared and their chemical stability pattern was determined. Some of the more promising compounds in this series were the vitamin A pivalate, α,α-dimethylvalerate, triethylacetate, α-methyl-α-ethylcaproate, and α,α-dimethylpalmitate. These were subjected to chemical evaluation tests such as oxidation, base-catalyzed hydrolysis, acid-catalyzed elimination, and acid-catalyzed isomerization (2, 3). It was then considered desirable to determine the biological availability of the esters that showed the best overall chemical stability when compared with the palmitate. Vitamin A α-methyl-α-ethylcaproate (2) and the α,α-dimethylpalmitate (3) were chosen, with cod liver oil and vitamin A palmitate functioning as standards. Two parameters were considered in the biological tests: (*a*) the amount of biological activity by a growth assay (4), and (*b*) the death rate in the various groups of rats, which was a further indication of the animals' ability to utilize the vitamin A ester (5).

EXPERIMENTAL

Chemical and spectrophotometric assays for vitamin A preparations may not give a true indication of the biological availability of vitamin A derivatives, especially new vitamin A derivatives that have unknown biological availability patterns. For this reason, a biological assay is very valuable in the final determination of potency.

The procedure used was a modification of the USP bioassay (5). It was based on the fact that the growth rate of a rat varies directly with the consumption of vitamin A (4). Vitamin A esters that are completely available will have higher growth rates than equimolar doses of esters that are not completely absorbed. Animals will die from avitaminosis A when fed derivatives that are poorly absorbed or not absorbed at all (5).

Table I—Average Weight (in Grams ±1 Standard Deviation) of Rats Fed Different Forms of Vitamin A

Days	Control Group	Cod Liver Oil Group	Vitamin A Palmitate Group	Vitamin A α-Methyl-α-ethylcaproate Group	Vitamin A α,α-Dimethylpalmitate Group
0	61	63	60	63	62
7	85	90	85	85	90
13	108	110	108	108	108
23	138	153	156	136	144
25	146 ± 13.5	165 ± 12.4	167 ± 9.2	141 ± 11.2	152 ± 9.2
30	144 ± 18.0	189 ± 14.3	187 ± 12.2	138 ± 18.1	167 ± 8.9
35	146 ± 14.2[a]	208 ± 15.4	210 ± 12.6	155 ± 16.9[b]	179 ± 12.8
38	156[c,d]	212 ± 8.9	216 ± 12.8	164 ± 14.0[e]	186 ± 13.8
41	157[d]	220 ± 10.2	232 ± 13.7	166 ± 17.0[e]	196 ± 11.6
45	144[d]	227 ± 10.8	242 ± 14.5	158 ± 24.3[e]	201 ± 12.9
52	128[d]	247 ± 11.5	264 ± 14.3	165 ± 26.4[a]	211 ± 15.8
55	—[f]	250 ± 11.1	271 ± 15.3	160 ± 27.8[g]	212 ± 18.8

[a] Represents 50% mortality. [b] Represents 30% mortality. [c] Represents 80% mortality. [d] No standard deviation was run in these groups because only one rat remained. [e] Represents 40% mortality. [f] Represents 100% mortality. [g] Represents 60% mortality.