of U factor by Ca^{2+} (Wojtczak and Lehninger, 1961). It can also be postulated that the proteolytic enzyme is directly inhibited by ATP, but the above explanation seems more plausible. Although the other proteolytic enzymes which stimulate pregnenolone synthesis were not examined in this respect, it is possible that some proteolytic enzymes constitute a new class of mitochondrial swelling agents.

It has been shown in previous studies that Ca^{2+} stimulates corticoid synthesis from endogenous precursors in rat adrenal homogenates (Koritz and Péron, 1959). This synthesis requires the presence of the large particles (pellet-2) and the supernatant, and of the two fractions pellet-2 is rate limiting (Péron and Koritz, 1960). In addition, Ca^{2+} has been shown to stimulate the synthesis of pregnenolone in isolated pellet-2 (Koritz, 1962). In view of these findings it was to be expected that any agent capable of stimulating the synthesis of pregnenolone in pellet-2 would also stimulate the overall synthesis of corticoids in adrenal whole homogenates. This exception is borne out by the data of Table III, where it is seen that all agents tested which stimulate pregnenolone synthesis also stimulate corticoid synthesis in the whole homogenate.

References

Birmingham, M. K., Elliot, F. H., and Valere, P. H. L. (1953), *Endocrinology 53*, 687.

Cleland, K. W. (1952), *Nature 170*, 492.

Elliot, F. H., Birmingham, M. K., Schally, A. V., and Schonbaum, E. (1954), *Endocrinology 55*, 721.

Grasset, E., and Schwartz, D. E. (1955), *Ann. Inst. Pasteur 88*, 271.

Halkerston, I. D. K., Eichhorn, J., and Hechter, O. (1961), *J. Biol. Chem. 236*, 374.

Hunter, F. E., and Ford, L. (1955), *J. Biol. Chem. 216*, 357.

Kimmel, J. R., and Smith, E. L. (1954), *J. Biol. Chem. 207*, 515.

Koritz, S. B. (1962), *Biochim. Biophys. Acta 56*, 63.

Koritz, S. B., and Hall, P. F. (1964), *Biochemistry, 3*, 1298.

Koritz, S. B., and Péron, F. G. (1959), *J. Biol. Chem. 234*, 3122.

Lehninger, A. L. (1959), *J. Biol. Chem. 234*, 2465.

Lehninger, A. L., and Remmert, L. F. (1959), *J. Biol. Chem. 234*, 2459.

Lever, J. D. (1956), *J. Biophys. Biochem. Cytol. 2 Suppl.*, No. 4, 313.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Péron, F. G., and Koritz, S. B. (1958), *J. Biol. Chem. 233*, 256.

Péron, F. G., and Koritz, S. B. (1960), *J. Biol. Chem. 235*, 1625.

Sabatini, D. D., De Robertis, E. D. P., and Bleichman, H. B. (1962), *Endocrinology 70*, 390.

Stone, D., and Hechter, O. (1954), *Arch. Biochem. Biophys. 51*, 457.

Wojtczak, L., and Lehninger, A. L. (1961), *Biochim. Biophys. Acta 51*, 442.

Antimetabolites of Mevalonic Acid. II. Inhibition of Ergosterol Synthesis in Yeast*

JOHN MORROW STEWART AND D. W. WOOLLEY

From the Rockefeller Institute, New York

Received July 27, 1964; revised October 5, 1964

The synthesis of three groups of new mevalonic acid analogs is described: 5-deoxy analogs, 5-methyl ethers, and aromatic analogs. Bioassay of these compounds and several previously prepared analogs showed that most of them inhibited growth of the mevalonic acid–requiring organism *Lactobacillus acidophilus*, and that certain members of each group caused partial inhibition of biosynthesis of ergosterol by yeast. The most active inhibitor of sterol synthesis was 3-hydroxy-3-methyl-5-phenyl-4-pentenoic acid, which caused 50% inhibition of ergosterol synthesis in yeast at a concentration (0.3 mg/ml) which did not inhibit growth. This compound, however, did not inhibit cholesterol synthesis when fed to growing mice.

Since the identification of mevalonic acid (3,5-dihydroxy-3-methylvaleric acid; MVA) as a key intermediate in the biosynthesis of sterols (Tavormina *et al.*, 1956), a number of investigators have sought to inhibit cholesterol biosynthesis by the use of structural analogs of MVA as possible antimetabolites. Mentzer *et al.* (1956) early found that 3-methyl-3-pentenoic acid would lower the liver cholesterol level of rats, while Gey *et al.* (1957) and Weiss *et al.* (1961) found that a number of compounds related structurally to MVA would inhibit the synthesis of cholesterol from MVA in rat liver homogenates. Several fluorinated analogs of MVA were investigated by Singer *et al.* (1959) and Kirschner *et al.* (1961); some compounds were found with high anti-MVA activity in enzyme systems. Daeniker and Druey (1960) found that certain derivatives of 5-thio MVA had a hypocholesteremic effect in rats. None of these assays, however, has provided accurate information about the effect of the analogs on sterol synthesis in an intact organism; the examination of serum cholesterol levels may give information primarily on transport, rather than synthesis, while *in vitro* enzyme assays may well not be subject to feedback and other control mechanisms which operate in the intact animal.

Intact organism assays are available for examination of the effects of MVA analogs on sterol synthesis and on other functions of this metabolite. Microbiological assays with sensitive strains of lactobacilli were used by Tamura *et al.* (1958) and by Stewart and Woolley (1959) to detect anti-MVA activity of a number of MVA analogs. Although the use of these organisms does not provide information on inhibition of sterol synthesis (since they do not synthesize sterols), the simplicity and great sensitivity of the *Lactobacillus* assay made it very useful during early stages of the investigation. Other intact organism assays which do involve sterol biosynthesis have been used (Stewart and

* Part I, *J. Am. Chem. Soc. 81*, 4951 (1959). An abstract of Part II appeared in *Federation Proc. 20*, 285 (1961).

TABLE I
ESTERS PREPARED BY THE REFORMATSKY REACTION

	Compound	Yield (%)	Bp, °C (μ Hg)	CCD System[a]	CCD K^b	Analyses Calcd. C	Calcd. H	Found C	Found H
I	Ethyl 3-hydroxy-3-phenylcaproate[c]	68	98 (20)			71.16	8.53	71.30	8.61
II	Ethyl 3-(*p*-biphenylyl)-3-hydroxyvalerate[d]	65	Mp 79–81[e]	0:6:4:1	1.5	76.48	7.43	76.27	7.58
III	Ethyl 3-hydroxy-3-(*p*-phenoxyphenyl)valerate[f]	46	Mp 47–49[e]			72.59	7.05	72.80	7.07
IV	Ethyl 3-hydroxy-3-methyl-5-phenyl-4-pentenoate[g]	74	122 (100)	1:3:3:1	0.68	71.77	7.74	72.05	7.78
V	Ethyl 2,3-dimethyl-3-hydroxy-5-phenyl-4-pentenoate[h]	70	115–117 (4)			72.55	8.12	73.07	8.31
VI	Ethyl 2,3-dimethyl-3-hydroxy-5-phenylvalerate[i]	74	118–120 (5)			71.97	8.86	72.01	8.95
VII	Ethyl 3-hydroxy-5-methoxy-3-methylvalerate[j]	35	102–106 (10 mm)			56.82	9.54	57.24	9.14
VIII	Ethyl 2,3-dimethyl-3-hydroxy-5-methoxyvalerate[k]	46	110–112 (10 mm)			58.80	9.87	58.92	9.74
IX	Ethyl 3,5-dimethyl-3-hydroxy-5-methoxycaproate[l]	41	48–50 (40)			60.52	10.16	59.18	9.52
X	Ethyl 3-(2-thienyl)crotonate[m]	64	72–74 (100)	1:4:3:1	1.9	61.21	6.17	60.77	6.61
XI	Ethyl 3-hydroxy-3-(1-naphthyl)butyrate[n]	72	Oil	2:5:5:2	1.1	74.39	7.02	74.41	7.11
XII	Ethyl 3-hydroxy-3-(2-naphthyl)butyrate[o]	70	Oil	2:5:5:2	1.0	74.39	7.02	74.41	6.96
XIII	Ethyl 3-hydroxy-3-methyl-5-*p*-chlorophenyl-4-pentenoate[p]	81	Oil	1:3:3:1	0.85	62.56	6.38	62.37	6.46
XIV	Ethyl 3-hydroxy-3-methyl-5-(1-naphthyl)-4-pentenoate[q]	75	Oil	1:3:3:1	2.1	76.03	7.09	76.46	6.86
XV	Ethyl 3-(2-acetoxyphenyl)crotonate[r]	39	110–113 (15)[s]			67.73	6.50	68.10	6.01
XVI	Ethyl 5-acetoxy-3-hydroxy-3-methyl-2-phenylvalerate[t]	42		2:5:4:2	0.67	65.29	7.53	65.33	7.53
XVII	Benzyl 5-acetoxy-3-hydroxy-3-methylvalerate[u]	27	70–75 (20)			64.27	7.19	64.34	7.29
XVIII	Ethyl 5-acetoxy-3-phenyl-2-pentenoate[v]	48	Mp 131–133[w]			68.68	6.92	68.53	6.59

[a] Volume ratio of the solvents: ethyl acetate–hexane–ethanol–water used for countercurrent distribution. [b] Partition ratio in the countercurrent distribution system given. [c] Prepared from butyrophenone and ethyl bromoacetate. [d] From 4′-phenylpropiophenone (Long and Henze, 1941) and ethyl bromoacetate. [e] Recrystallized from hexane. [f] From 4′-phenoxypropiophenone (XXXVIII) and ethyl bromoacetate. [g] From benzalacetone and ethyl bromoacetate. This compound was prepared by Kohler and Heritage (1910), who reported bp 192° at 20 mm Hg. [h] From benzalacetone and ethyl 2-bromopropionate. [i] From 1-phenyl-3-butanone (Smith, 1914) and ethyl 2-bromopropionate. [j] From 1-methoxy-3-butanone (Killian *et al.*, 1936) and ethyl bromoacetate. [k] From 1-methoxy-3-butanone and 2 moles ethyl 2-bromopropionate in benzene alone as solvent. [l] From 2-methoxy-2-methyl-4-pentanone (we thank Shell Chemical Company for a generous gift of this compound) and ethyl bromoacetate in benzene alone. [m] From 2-acetothienone and ethyl bromoacetate. [n] From 1-acetonaphthone and ethyl bromoacetate. [o] From 2-acetonaphthone and ethyl bromoacetate. [p] From 1-(*p*-chlorophenyl)-2-buten-3-one (Von Walther and Raetze, 1902) and ethyl bromoacetate. [q] From 1-naphthalacetone (Gibson *et al.*, 1926) and ethyl bromoacetate. [r] From 2-acetoxyacetophenone (Friedlander and Neudorfer, 1897) and ethyl bromoacetate. Much unreacted ketone (22%) was recovered. [s] Mp 76–79°. [t] From 1-acetoxy-3-butanone (Cornforth *et al.*, 1959) and ethyl 2-bromo-2-phenylacetate (Anschutz, 1907). [u] From 1-acetoxy-3-butanone and benzyl bromoacetate. [v] From β-acetoxypropiophenone (von Auwers and Müller, 1933) and ethyl bromoacetate. [w] From ethyl acetate–hexane.

TABLE II. ACIDS AND LACTONES

	Compound	Prepd. from	Yield (%)	Mp	CCD System[a]	CCD K^b	Calcd. C	Calcd. H	Found C	Found H
XIX	3-Hydroxy-3-phenylcaproic acid	I	79	120–121[c]			69.21	7.75	69.56	7.78
XX	3-(*p*-Biphenylyl)-3-hydroxyvaleric acid	II	70	162–165[d]			75.53	6.71	75.60	6.83
XXI	3-Hydroxy-3-(*p*-phenoxyphenyl)valeric acid	III	96	119–120[e]			71.31	6.34	71.50	6.27
XXII	3-Hydroxy-3-methyl-5-phenyl-4-pentenoic acid	IV	45	Oil	2:2:2:1	1.0	69.88	6.84	70.38	6.99
XXIII	2,3-Dimethyl-3-hydroxy-5-phenyl-4-pentenoic acid	V	72	Oil	2:2:2:1	0.77	70.89	7.32	71.14	7.38
XXIV	3-Hydroxy-3-methyl-5-phenylvaleric acid	/	55	83–86[e]			69.21	7.74	69.36	7.84
XXV	2,3-Dimethyl-3-hydroxy-5-phenylvaleric acid	VI	80	Oil	2:2:2:1.2	1.1	70.24	8.16	70.07	8.02
XXVI	3-Hydroxy-3-methyl-5-*p*-chlorophenyl-4-pentenoic acid	XIII	35	Oil	2:3:2:2	1.3	59.89	5.44	60.18	5.45
XXVII	3-Hydroxy-3-methyl-5-(1-naphthyl)-4-pentenoic acid	XIV	30	Gum	1:2:2:1	0.33	74.98	6.29	75.45	6.28
XXVIII	2-Benzylidenecyclohexanol-1-acetic acid	"	75	123–126[e]	1:2:2:1	0.47	73.14	7.37	73.03	7.49
XXIX	3-(2-Thienyl)crotonic acid	X	74	113–115[h]			57.14	4.80	56.91	5.00
XXX	3-Hydroxy-3-(1-naphthyl)butyric acid	XI	78	140–141[c]			73.02	6.13	73.46	6.30
XXXI	3-Hydroxy-3-(2-naphthyl)butyric acid	XII	85	101–103[c,i]			73.02	6.13	72.90	6.17
XXXII	3-Hydroxy-5-methoxy-3-methylvaleric acid	VII	45	Oil	4:1:0:4	0.32	51.84	8.70	51.25	8.98
XXXIII	2,3-Dimethyl-3-hydroxy-5-methoxyvaleric acid	VIII	55	Oil	4:1:0:4	0.77	54.53	9.15	54.20	8.63
XXXIV	3,5-Dimethyl-3-hydroxy-5-methoxycaproic acid	IX	70	Oil	1:1:1:1	0.64	56.82	9.54	56.51	9.77
XXXV	3-(2-Hydroxyphenyl)crotonic acid	XV	85	150–151[j]			67.40	5.66	67.40	5.82
XXXVI	3-Hydroxy-3-methyl-2-phenyl-5-valerolactone	XVI	45	65–67[k]	1:1:1:1	1.70	69.88	8.64	69.99	8.01
XXXVII	3-Phenyl-2-penteno-5-lactone	XVIII	65	56–58	1:1:1:1	1.10	75.84	5.79	75.53	6.16

[a] See footnote *a* of Table I. [b] See footnote *b* of Table I. [c] Recrystallized from benzene-hexane. [d] From ethanol. [e] From benzene. [f] Prepared by saponification of ethyl 3-hydroxy-3-methyl-5-phenylvalerate (Canonica *et al.*, 1954). [g] Prepared by saponification of crude ethyl 2-benzylidenecyclohexanol-1-acetate, which was in turn prepared from 2-benzylidenecyclohexanone (Vorlander and Kunze, 1926) and ethyl bromoacetate. [h] From hexane. [i] Melted partially at 71–73°, then resolidified. [j] From ethyl acetate-hexane. [k] From ether-petroleum ether.

Woolley, 1959), such as the biosynthesis of ergosterol by growing yeast cultures, and total body cholesterol synthesis by young growing mice.

The first paper of this series (Stewart and Woolley, 1959) described the synthesis of a number of structural analogs of MVA and the bioassay of those compounds for their anti-MVA potency. The compounds described there were homologs of MVA with longer or shorter carbon chains, and analogs bearing alkyl substituents on the chain. Many of those compounds were found to act as antimetabolites of MVA when assayed for their ability to inhibit the growth of *Lactobacillus acidophilus*, but none was found to inhibit sterol biosynthesis, either in yeast or in intact mice.

The present paper describes the synthesis and assay of a further series of analogs of MVA, some of which were able to inhibit the synthesis of ergosterol in growing yeast. The types of structural modifications incorporated into these analogs were: replacement of the 5-hydroxyl by hydrogen, dehydration to produce a 4,5-double bond, methylation of the 5-hydroxyl, introduction of alkyl groups, introduction of aromatic groups, and combinations of these. A common feature of these analogs was the lack of a free hydroxyl group in position 5. Since the first step in the conversion of MVA to sterols is the phosphorylation of the 5-hydroxyl, the absence of this function in the analogs might lead to effective antimetabolites. Methods used for the synthesis and assay of these compounds were similar to those previously described (Stewart and Woolley, 1959).

EXPERIMENTAL

Melting points were determined in capillaries. Infrared spectra were determined on all compounds and were consistent with the assigned structures. The hexane used was a redistilled petroleum ether fraction, bp 60–70°.

Preparation of Esters by the Reformatsky Reaction.—The procedure of Hoffman *et al.* (1957) was used, except that the reaction medium was a mixture of equal parts of benzene and ether. The products were purified by fractional distillation through a Vigreux column or by countercurrent distribution (100 transfers or more in the systems shown in Table I). Properties of the esters are given in Table I.

Saponification of Esters.—Procedure B of the previous paper (Stewart and Woolley, 1959) was used to avoid dehydration. The acids and lactones thus obtained were purified by countercurrent distribution or crystallization. Properties of the acids and lactones are given in Table II.

4'-Phenoxypropiophenone (XXXVIII).—A solution of 34.0 g of diphenyl ether and 18.6 g of propionyl chloride in 75 ml of carbon disulfide was added slowly to a stirred mixture of 29.2 g of aluminum chloride and 75 ml of carbon disulfide. After the addition was complete, the mixture was heated under reflux for 4 hours. The carbon disulfide was evaporated, the residue was poured into ice and water, and the product was extracted into ethyl acetate. The ethyl acetate solution was washed with water and 0.5 M sodium bicarbonate solution and dried over magnesium sulfate. The solvent was evaporated and the product was fractionated *in vacuo* to yield 30.3 g (67%) of the desired ketone, bp 130–131° (7 μ Hg). For analysis a sample was recrystallized from ether-hexane, mp 38–39°.

Anal. Calcd for $C_{15}H_{14}O_2$: C, 79.62; H, 6.24. Found: C, 79.28; H, 6.47.

N,N-Bis-2-hydroxyethylamide of 3-Hydroxy-3-methyl-5-phenyl-4-pentenoic acid (XXXIX).—A mixture of

2.0 g of ethyl 3-hydroxy-3-methyl-5-phenyl-4-pentenoate (IV) and 5.0 g of diethanolamine was heated at 140° for 2 hours, and the product was purified by countercurrent distribution (100 transfers) in the system ethyl acetate–hexane–ethanol–water (3:1:1:3). The pure amide (k 1.0) weighed 2.0 g (82%), and was a gum.

Anal. Calcd for $C_{16}H_{23}NO_4$: C, 65.51; H, 7.90; N, 4.78. Found: C, 64.90; H, 7.78; N, 4.68.

Benzyl 5-Acetoxy-3-methyl-3-(2-tetrahydropyranyloxy)-valerate (XL).—A mixture of 12.2 g of benzyl 5-acetoxy-3-hydroxy-3-methylvalerate (XVII) and 7.5 g of dihydropyran was treated with 2 drops of concentrated hydrochloric acid and allowed to stand at room temperature for 24 hours. The product was dissolved in ethyl acetate, and the solution was washed with water, sodium bicarbonate solution, and water, and dried over magnesium sulfate. After evaporation of the solvent under reduced pressure, the residue was purified by countercurrent distribution for 100 transfers in the system ethyl acetate–hexane–ethanol–water (1:4:3:1). The pure product (k 1.1) was a colorless oil. Upon distillation at 0.02 mm Hg, the reaction was reversed and the starting ester (XVII) was obtained.

Anal. Calcd for $C_{10}H_{28}O_6$: C, 65.91; H, 7.74. Found: C, 66.19; H, 7.93.

Yeast Assay.—*Saccharomyces cerevisiae* (Hanson strain No. 1) was grown in a medium like that of Woolley and White (1943) except that magnesium sulfate and calcium chloride were present at 125 mg per liter and thiamine at 0.1 mg per liter, and potassium iodide was omitted. Solutions of the compounds to be tested were sterilized by filtration and added to autoclaved basal medium. Water-insoluble compounds were added as their sodium salts. For compounds XXII, XXIII, XXVIII, XXX, and XXXI, ethanolic solutions of the acids were made alkaline with potassium hydroxide solution, diluted with water, evaporated sufficiently under reduced pressure to remove the alcohol, and adjusted to pH 6.5 and a concentration not greater than 1 mg/ml. The organisms were grown in 50-ml portions of medium in 250-ml stationary erlenmeyer flasks at 30° for 24 hours. Dry weight of the cells obtained was measured by comparison of the turbidity of the grown cultures with a standard curve. The cells were killed by heating the culture just to boiling and were harvested by centrifugation. They were washed with water and then were soaked for one hour in methanol-water (3:1) to remove interfering substances. Ergosterol was then extracted by soaking the cells for 20 hours at room temperature in the dark in 15 ml of ethanol-benzene (2:1). After centrifugation duplicate aliquots of the supernatant solution (2 and 4 ml) were pipetted into tubes and evaporated to dryness in a stream of warm air. Ergosterol was measured in two different ways. In the first pair of tubes, the Liebermann-Burchard method, using 8 ml of the reagent of Abell *et al.* (1952), was used. Absorbance at 420 mμ was measured after the tubes had stood for 30 minutes at 25°. The residue in the other pair of tubes was dissolved in 4 ml of redistilled 95% ethanol, and ergosterol was calculated directly from the absorbance of the solution at 282 mμ. Use of the two methods showed whether what was being measured was ergosterol alone or included interfering materials. Inhibition of growth and of ergosterol synthesis caused by the most active compound (XXII) is shown in Table III. Results with all of the analogs are summarized in Table IV.

The buffered medium used for growing yeast (see discussion) was like that described above, except that the ammonium sulfate was replaced by ammonium acetate (4.75 g/liter), potassium dihydrogen phosphate was reduced to 1 g/liter, dipotassium hydrogen phosphate was added (1 g/liter), and calcium chloride was eliminated.

TABLE III
EFFECT OF COMPOUND XXII ON GROWTH AND ERGOSTEROL SYNTHESIS IN YEAST

Analog Added (mg/ml)	Culture Growth[a] (mg)	Ergosterol Concentration[b] (μg/mg)
0.0	170	5.3
0.02	160	4.2
0.1	160	2.4
0.2	120	2.2
0.4	53	2.5

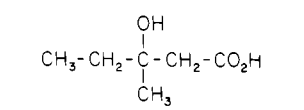
[a] Dry weight of cells produced in 50 ml of a 24-hr culture. [b] Micrograms of ergosterol per milligram of dry cell weight measured by the Liebermann-Burchard reaction.

Lactobacillus Assay.—Compounds were tested for their ability to inhibit the growth of *Lactobacillus acidophilus* ATCC 4963 in the presence of mevalonic acid by the procedure previously described (Stewart and Woolley, 1959). Results of the *Lactobacillus* assay are shown in Table IV.

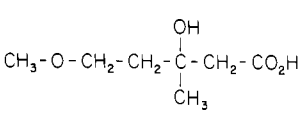
Mouse Assay.—Compound XXII and 2-methyl-MVA were also tested for their ability to inhibit total formation of cholesterol by young growing mice by the method previously described (Stewart and Woolley, 1959). The compounds were administered orally as 1% of the diet. Total carcass cholesterol determinations performed after two weeks' administration of the analogs showed no significant difference between control and analog-treated groups of animals.

DISCUSSION

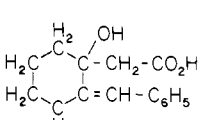
Several of the compounds synthesized in the present group of MVA analogs were found to be effective inhibitors of ergosterol synthesis by yeast. The most active of these was 3-hydroxy-3-methyl-5-phenyl-4-pentenoic acid (XXII), which caused a maximum of 50% inhibition of ergosterol synthesis at a level of analog (0.1 mg/ml) which was without effect on the growth of the organism. Higher levels of analog never completely suppressed sterol synthesis, even at levels which caused inhibition of growth. This failure to obtain complete inhibition of ergosterol synthesis was characteristic of all of the active compounds. Only one other compound was found to cause as much as 50% inhibition of ergosterol synthesis, namely, 2,3-dimethyl-3-hydroxyvaleric acid (2-methyl-DMVA). With this compound, however, a concentration 20 times as high as that of XXII was required to produce



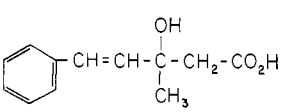
Deoxymevalonic Acid (DMVA)



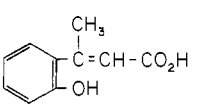
XXXII



XXVIII



XXII



XXXV

TABLE IV
BIOLOGICAL EFFECTS OF THE ANALOGS

Compound	*L. acidophilus*		*S. cerevisiae*	
	Inhib. Concn (mg/ml)[a]	Type of Inhib.[b]	Growth Inhib. Concn (mg/ml)[c]	Effect on Sterol Synth.[d]
2-Methyl MVA[e]	0.007	R,C	NE	NE
Deoxy Analogs				
DMVA[f]	0.3	IR	NE	30% I at 1.0 mg/ml
2-Methyl DMVA[g]	0.7	IR	NE	50% I at 4.0
4-Methyl DMVA[h]	0.8	IR	NE	25% I at 4.0
5-Methyl DMVA[i]	0.6	IR	NE	NE
Cyclopentanol-1-acetic acid[j]	0.5		S	NE
Cyclohexanol-1-acetic acid[k]	>0.5		>2	S
Aromatic Deoxy Analogs				
3-Hydroxy-3-phenylbutyric acid[l]	0.5	R,C	NE	NE
3-Hydroxy-3-phenylvaleric acid[m]	NE		>4.0	S
XIX	NE		2.0	S
XX	0.07		0.7	S
XXI	0.07		1.0	S
XXII	NE		0.3	50% I at 0.1 mg/ml[n]
XXIII	NE		1.5	35% I at 1.0
XXIV	0.3	NE	NE	NE
XXV	NE		>4	S
XXVI	0.5		0.2	S
XXVII	0.1		S	NE
XXVIII	>0.5		0.2	NE
XXIX	0.3		0.02	NE
XXX	0.5		4.0	S
XXXI	>0.5		0.2	S
XXXV, XXXVI, XXXVII, XXXIX, and XL were without effect on either organism.				
Ether Analogs				
VII			0.2	S
XXXII	0.6		S	25% I at 1.0
XXXIII	0.6		>2.0	NE
XXXIV	NE		NE	30% I at 1.0
Miscellaneous Compounds				
SK & F 525[o]	0.07	IR	0.1	NE
SK & F 3301[p]	0.03	IR	0.5	NE
SK & F 7997[q]	NE		>5.0	S

[a] The concentration of analog which gave half-maximal inhibition of growth in the presence of 0.05 μg/ml of DL-MVA lactone. NE = no effect. [b] C = competitive, R = reversible, IR = irreversible. [c] The concentration of analog which caused 50% inhibition of normal yeast growth. S = stimulation, NE = no effect. [d] NE = no effect, S = stimulation, I = inhibition. The inhibitions recorded are the maximum obtainable with any amount of analog. [e] Tamura and Takai (1957). [f] Deoxy MVA, 3-hydroxy-3-methylvaleric acid (Pocrowsky, 1900). [g] Abbott *et al.* (1928). [h] Schryver (1893). [i] Semljanitzin (1881). [j] Kon and Linstead (1925). [k] Schwenk and Papa (1945). [l] Arbuzov (1901). [m] Posner (1910). [n] This level of analog had no significant effect on growth. [o] Diethylaminoethyl ester of 2,2-diphenylvaleric acid. We thank Dr. W. L. Holmes of Smith, Kline and French Laboratories for a gift of these compounds. [p] Dimethylaminoethyl 2,2-diphenylamyl ether. [q] Tris(diethylaminoethyl) phosphate.

the effect. Five other compounds were also found to be able to inhibit sterol synthesis in yeast without inhibition of growth, but none of them caused more than 35% reduction of sterol synthesis. Active compounds were found among all three groups investigated, namely, aromatic analogs, 5-deoxy analogs, and 5-methyl ethers.

The structural features necessary for inhibition of ergosterol synthesis were clarified by investigation of a series of related compounds. In the most active compound (XXII) the structure of MVA has been modified by dehydration to give a 4,5-double bond and addition of a phenyl group to the 5-position. Since 2-methyl-MVA had earlier been found (Tamura *et al.*, 1958) to be an effective anti-MVA in lactobacilli (cf. Table IV), a methyl group was added to the 2-position of XXII. The compound thus synthesized, XXIII, was found to have lowered, rather than enhanced, ability to inhibit ergosterol synthesis. The effect of saturation of the 4,5-double bond in these two analogs was studied by preparation and assay of the corresponding saturated compounds XXIV and XXV; they were found to be devoid of ergosterol-inhibiting activity. Structural specificity for the aromatic group in the 5-position was investigated in compounds XXVI and XXVII. Here it was found that exchange of the phenyl group of compound XXII for either *p*-chlorophenyl (XXVI) or 1-naphthyl (XXVII) caused complete loss of activity in the yeast assay.

An investigation of cyclic analogs involved the incorporation of part of the carbon chain of MVA into a six-membered ring. Compound XXII was cyclized by the incorporation of carbons 3 and 4 and the methyl group carbon into a cyclohexane ring, to give compound XXVIII. The lack of effectiveness of this compound in inhibition of ergosterol synthesis would make it appear that the conformation imposed on the molecule by this cyclization does not conform to the steric requirements of the active centers of the enzymes that utilize MVA in yeast. A further cyclic analog of MVA, XXXV, which has carbons 4 and 5 incorporated into a benzene ring, was also found to lack biological activity.

Although the 5-phenyl group and the 4,5-double bond individually seemed to be crucial for the high activity

of compound XXII, simultaneous elimination of both these features gave an active compound, 5-deoxymevalonic acid (DMVA). In a series of compounds related to DMVA it was found that the biological effect was enhanced by the addition of a methyl group in the 2-position (2-methyl-DMVA). 4-Methyl-DMVA was similar in biological activity to DMVA itself, while 5-methyl-DMVA was devoid of any ability to inhibit ergosterol synthesis. The enhancing effect of the added methyl group in position 2 of DMVA might suggest that these deoxy analogs were exerting their effect at a different point in the sterol biosynthetic pathway than was compound XXII, since with that compound the addition of a 2-methyl substituent diminished the anti-MVA potency.

The 5-methyl ether of MVA (XXXII) and the related compound XXXIV, which had two additional methyl groups on the chain at position 5, were both found to be inhibitors of ergosterol synthesis. This activity was completely lost in the closely related compound XXXIII, which differed from MVA 5-methyl ether only in the addition of a methyl group at position 2.

A free carboxyl group seemed to be necessary for inhibition of ergosterol synthesis. Conversion of XXII into its amide with diethanolamine (XXXIX) yielded an inactive compound. Similarly, the ester (VII) of MVA 5-methyl ether (XXXII) was inactive, whereas the acid was active.

The concentration of ergosterol found in 24-hour yeast cultures varied inversely with the amount of cell growth during that period. When growth was partially inhibited by toxic compounds, the concomitant increase in ergosterol concentration sometimes amounted to 300% of the normal (12 mg/g dry weight instead of the usual 3–5 mg), although the total amount of ergosterol synthesized by a 50-ml culture was much reduced. This increased ergosterol concentration was seen with most toxic compounds, and is not regarded as necessarily being related to anti-MVA action. It is well known that yeast and other fungi synthesize much more ergosterol when kept on a depleted medium than when actively growing in an adequate medium (Halden *et al.*, 1933). Most of the aromatic analogs assayed caused some inhibition of growth, although with the active compounds this inhibition was seen only at levels higher than were necessary to cause inhibition of sterol synthesis.

The effectiveness of the active analogs in inhibition of ergosterol synthesis depended upon the *p*H of the medium in which the yeast was grown. The medium used for growth of yeast contained ammonium sulfate as the nitrogen source and had an initial *p*H of 5.5. As the culture grew and depleted the ammonia, the sulfuric acid thus liberated caused the *p*H of the medium to drop to 3.0 after 24 hours. The values for inhibition of ergosterol synthesis reported in Table IV were obtained under these conditions. Some difficulty was encountered in the assay of certain of the aromatic analogs in this system, since at high analog concentrations the lowered *p*H of the medium sometimes caused the free acid to precipitate and thus interfere with turbidity measurements of the cultures. For this reason the buffered medium described in the experimental part was devised. A 24-hour culture of yeast in this medium did not show a *p*H below 5.5. None of the compounds found to inhibit ergosterol biosynthesis in the normal medium was capable of doing so in this buffered medium. The exact cause of this phenomenon is unknown, but the explanation may be that acidic analogs were unable to penetrate into the yeast cell. With the normal medium, the rapidly attained *p*H of 3.0 is sufficiently below the *pK* of most carboxylic acids to allow them to exist largely in the undissociated form which would be able to penetrate the lipid barrier of the cell wall without a specific transport mechanism.

There appeared to be relatively little correlation between the effectiveness of analogs as inhibitors of ergosterol synthesis in yeast and their ability to inhibit the growth of *L. acidophilus*. Nearly all of the analogs inhibited the MVA-dependent growth of *L. acidophilus*, but the most active of these (XX, XXI, XXVII, 2-methyl-MVA) had no effect on ergosterol synthesis. Conversely, some of the most active inhibitors of ergosterol synthesis (XXII, XXIII, XXXIV) were without effect on the growth of *L. acidophilus*. The only group of compounds where some correlation between the two types of activity was found was the deoxy analogs. In these, the irreversible nature of their anti-MVA activity in *L. acidophilus* might well be related to their potency in inhibition of ergosterol synthesis by yeast. Evidently there exist great differences in the enzyme specificities or the control mechanisms operating in these two organisms.

Metabolic differences among species were further pointed up by the failure of the two most active compounds in the microbial assays to inhibit cholesterol synthesis in young growing mice. Similarly, compound XXXII (MVA 5-methyl ether), which inhibited ergosterol synthesis in the present study, was found by Humber *et al.* (1963) to have no effect on the incorporation of MVA into cholesterol in a rat liver homogenate system. These data would all indicate that effects of MVA analogs in mammalian systems cannot be inferred from microbiological assays.

The three Smith, Kline and French compounds listed in Table IV were tested because they have been reported to have a hypocholesteremic effect in animals (Holmes, 1964), and because two of them may be regarded as analogs of MVA. These two, SK & F 525 and SK & F 3301, were found to be quite potent, but irreversible, inhibitors of *L. acidophilus* growth. The failure of MVA to reverse this growth inhibition made it uncertain whether their toxic action on this organism was due to anti-MVA activity. However, neither compound inhibited ergosterol synthesis in yeast. Holmes and DiTullio (1962) have shown that in mammalian liver homogenates these compounds inhibit the sterol synthetic pathway at points involving MVA phosphates and polyprenol phosphates. SK & F 7997 was without significant activity in either microbial assay. Holmes has shown that this compound inhibits the conversion of lanosterol to zymosterol in mammalian systems.

The importance of the reversibility by MVA of the action of the analogs requires some comment. An irreversible analog would be able to block the incorporation of MVA into isoprenoid compounds in a way such that excess MVA could not overcome the effects of the analog. The production of MVA by yeast, for example, must be quite large because the amount of it required to make the ergosterol contained in the yeast is quite considerable. For example, a full-grown culture has metabolized 40 μg of MVA/ml to produce the 20 μg/ml of ergosterol it contains. The same consideration applies to mammals which are making cholesterol. A growing mouse must produce at least 5 mg of MVA per day to account for the cholesterol it synthesizes. Because the MVA production is so large, a successful antimetabolite of it either must have a very low inhibition index or else must be an irreversible antagonist. By contrast, in the *L. acidophilus* assay, a reversible analog can still show good activity at low levels because the organism is not synthesizing MVA and is restricted to the very small amount (0.025 μg/ml) added to the medium. The degree of activity required in the three

cases is of a different order of magnitude. Considerations such as these may be the basis of the finding in the present work that, although a reversible antimetabolite such as 2-methyl-MVA showed very high activity in the *L. acidophilus* assay, it was unable to inhibit ergosterol synthesis in yeast. On the other hand, an irreversible analog such as 2-methyl-DMVA was a potent inhibitor of ergosterol synthesis even though it was 100 times less active than 2-methyl-MVA in the *L. acidophilus* assay. One of the aims in the design of the present series of analogs was to realize such irreversible antimetabolites. The experimental findings may be taken to indicate the importance of obtaining irreversible antagonists.

ACKNOWLEDGMENT

The authors thank Mrs. Susanna Ander and Mr. David Greenseid for technical assistance, and Mr. S. T. Bella for the microanalyses.

REFERENCES

Abbott, A. E., Kon, G. A. R., and Satchell, R. D. (1928), *J. Chem. Soc.* 2514.
Abell, L. L., Levy, B. B., Brodie, B. B., and Kendall, F. E. (1952), *J. Biol. Chem. 195*, 357.
Anschutz, R. (1907), *Ann. 354*, 117.
Arbuzov, A. (1901), *J. Russ. Phys. Chem. Soc. 33*, 38; *Chem. Centr.*, 998 (1901).
Auwers, von, K., and Müller, H. (1933), *J. Prakt. Chem. 137*, 102.
Canonica, L., Fiecchi, A., and Adobbati, M. (1954), *Rend. Ist. Lombardo Sci. 87*, 356; *Chem. Abstr. 50*, 6377 (1956).
Cornforth, J. W., Cornforth, R. H., Pelter, A., Horning, M. G., and Popjac, G. (1959), *Tetrahedron 5*, 311.
Daeniker, H. V., and Druey, J. (1960), *Helv. Chim. Acta 43*, 983.
Friedlander, P., and Neudorfer, J. (1897), *Ber. 30*, 1077.
Gey, K. F., Pletscher, A., Isler, O., Ruegg, R., and Würsch, J. (1957), *Helv. Chim. Acta 40*, 2354.
Gibson, C. S., Hariharan, K. V., Menon, K. N., and Simonsen, J. L. (1926), *J. Chem. Soc.* 2247.
Halden, W., Bilger, F., and Kunze, R. (1933), *Naturwiss. 21*, 660.
Hoffman, C. H., Wagner, A. F., Wilson, A. N., Walton, E., Shunk, C. H., Wolf, D. E., Holly, F. W., and Folkers, K. (1957), *J. Am. Chem. Soc. 79*, 2316.
Holmes, W. L. (1964), *in* Lipid Pharmacology, R. Paoletti, ed., New York, Academic, p. 132.
Holmes, W. L., and DiTullio, N. (1962), *Am. J. Clin. Nutr. 10*, 310.
Humber, L. G., Kraml, M., Dubuc, J., and Gaudry, R. (1963), *J. Med. Chem. 6*, 210.
Killian, D. B., Hennion, G. F., and Nieuwland, J. A. (1936), *J. Am. Chem. Soc. 58*, 892.
Kirschner, K., Henning, U., and Lynen, F. (1961), *Ann. 644*, 48.
Kohler, E. P., and Heritage, G. L. (1910), *Am. Chem. J. 43*, 475.
Kon, G. A. R., and Linstead, R. P. (1925), *J. Chem. Soc. 127*, 616.
Long, L. M., and Henze, H. R. (1941), *J. Am. Chem. Soc. 63*, 1937.
Mentzer, C., Zwingelstein, G., and Jouanneteau, J. (1956), *Compt. Rend. Acad. Sci. 242*, 943.
Pocrowsky, A. (1900), *J. Prakt. Chem.* (2), *62*, 301.
Posner, T. (1910), *J. Prakt. Chem.* (2) *82*, 425.
Schryver, S. B. (1893), *J. Chem. Soc. 63*, 1327.
Schwenk, E., and Papa, D. (1945), *J. Am. Chem. Soc. 67*, 1432.
Semljanitzin, A. (1881), *J. Prakt. Chem.* (2) *23*, 263.
Singer, F. M., Januszka, J. P., and Borman, A. (1959), *Proc. Soc. Exptl. Biol. Med. 102*, 370.
Smith, C. (1914), *J. Chem. Soc. 105*, 1703.
Stewart, J. M., and Woolley, D. W. (1959), *J. Am. Chem. Soc. 81*, 4951.
Tamura, S., and Takai, M. (1957), *Bull. Agr. Chem. Soc. Japan 21*, 394.
Tamura, S., Tamura, G., Takai, M., Nakamura, S., and Shiro, T. (1958), *Bull. Agr. Chem. Soc. Japan, 22*, 202.
Tavormina, P. A., Gibbs, M. H., and Huff, J. W. (1956), *J. Am. Chem. Soc. 78*, 4498.
Vorlander, D., and Kunze, K. (1926), *Ber. 59*, 2078.
Von Walther, R., and Raetze, W. (1902), *J. Prakt. Chem.* (2) *65*, 258.
Weiss, H., Schiffmann, E., and Titus, E. (1961), *J. Lipid Res. 2*, 258.
Woolley, D. W., and White, A. G. C. (1943), *J. Exptl. Med. 78*, 489.

Interrelationship of Sterol and Fatty Acid Biosynthesis in Rat Liver Slices as Related to Dietary Lipid

E. R. DILLER AND O. A. HARVEY

From the Biology-Pharmacology Research Division, Lilly Research Laboratories, Indianapolis, Indiana

Received March 31, 1964; revised August 10, 1964

In vitro studies on cholesterol and fatty acid biosynthesis were made in liver slices from rats maintained on four levels of dietary fat. With [1-^{14}C]acetate as precursor, cholesterol biosynthesis was found to be directly related to the fat content of the diet, whereas an inverse relationship was found in lipogenesis. In a more detailed study on the sterol biosynthetic pathway with [2-^{14}C]mevalonate, the incorporation of this precursor into isoprenols, squalene, and cholesterol was determined. Sterol biosynthesis from mevalonate was found to be related to the amount of dietary lipid. The incorporation of the precursors into sterol was found to be directly related to the hepatic sterol levels.

Within recent years the effect of dietary lipids on cholesterol metabolism has been of great interest (Portman and Stare, 1959). However, only a few reports have considered the relationship of dietary fat to endogenous cholesterol metabolism. Endogenous cholesterol was found to accumulate in the livers of male rats fed a fat-free diet, whereas the administration of appropriate amounts of fat led to normal levels (Alfin-Slater *et al.*, 1954). On the other hand, in both the rat (Klein, 1959) and the rabbit (Diller *et al.*, 1961) endogenous cholesterol levels in the liver and blood are directly related to the concentration of fat in the diet.

If the animals in these experiments were in a steady