Current Trends in the Development of Sterol Biosynthesis Inhibitors: Early Aspects of the Pathway

The following review article was written by Thomas J. Bach of the Botanical Institute (Plant Physiology and Plant Biochemistry), University of Karlsruhe, Kaiserstr. 12, D-7500 Karlsruhe, West Germany. It was done on the request of W. David Nes of the U.S. Department of Agriculture's Richard B. Russell Agricultural Research Center, who serves as Associate Editor for JAOCS *News for Plant Lipids.*

INTRODUCTION

In prokaryotes and eukaryotes, notably in vascular plants, an array of isoprenoids (also referred to as isopentenoids) (1,2) and isoprenoid-derivatives of major and minor importance for cellular function are synthesized (1-3), although not necessarily continuously, throughout ontogenetic development.

The elucidation of biochemical pathways leading to isoprenoid end-products has been a matter of intense research over many years (1-5). In the fields of enzymology and regulation, sequencing continues to be pursued aggressively. The physiological importance of sterols as essential components of eukaryotic biomembranes as well as their metabolic role, e.g., in the synthesis of steroid hormones, also has been intensively investigated. Cholesterol, the typical membrane-sterol constituent of animal membranes, is the most acclaimed small molecule in nature if for no other reason than, as Brown and Goldstein (6) have stated, the vast number of Nobel prizes awarded to scientists whose work has centered around problems associated with its chemistry, biosynthesis, distribution and function. The typical sterol of more advanced fungi is ergosterol (2), and sitosterol is the usual sterol isolated from vascular plants. Cholesterol, meanwhile, is present not only in less-advanced fungal groups (7,8) but is distributed generally throughout plants from cyanophytic bacteria to tracheophytes (2).

There is much evidence for sterols having at least two functions in mediating growth (9,10) and another that may govern reproduction (11-13) of the fungal life cycle (7). The multiple functions have been defined operationally by quantitative findings related to structure-activity comparisons. One is to act as an architectural component of the membrane (also called "bulk function") and the other is characterized as having a regulatory, sparking or synergistic role (10). Very recently, Popják and Nes have shown that cholesterol and biogenetically related sterols of mammalian (14,15), fungal (16) and plant (17) origin are localized in the nucleus bound to chromatin (14), and this trace-level sterol must be renewed biosynthetically for the cells to proliferate (14-17). Presumably, sterol-protein binding is the regulatory agent (7,18).

The knowledge that select sterols, rather than "any sterol," are essential to cell vitality permits the development of bioregulators, albeit synthetic or natural, to alter sterol biosynthesis. Sterol biosynthesis inhibitors (SBI) possess a myriad of potential uses in medicine and agriculture such as in preventing the induction of the disease cycle of pathogenic fungi. Illustrating sophisticated biochemical subtleties, some SBI make use of gentle differences in the properties of key regulatory enzymes and thus are designed with the enzymes' stereochemistry in mind. Others make use of kinetic differences in their ability to be taken up by the cell or whole organism to be transported in situ from one subcellular compartment to another and to be metabolized.

In mammalian physiology, SBI possess clinical application. For instance, elevated serum cholesterol levels in humans are known to contribute to atherosclerosis, the leading health-risk factor in many developed countries (6). SBI are given to patients with this disease. It appears noteworthy that the development of compounds or the search for antibiotics being active in down-regulation of mammalian cholesterol synthesis frequently has failed because of toxic side effects (19,20). However, those drugs frequently serve as parental compounds in the development of fungicides or herbicides having SBI properties (21-23). Several excellent and comprehensive reviews on sterol biosynthesis or on SBI and related subjects (4,21-23) have been published. This article will discuss a modern approach of using biological systems in which a lipid pathway—the steroid pathway—has been manipulated specifically through changes in genetic, biochemical or cultural conditions. Due to space limitations, this article will examine only sterolic enzymes in the early part of the pathway so far demonstrably affected by SBI.

INHIBITION OF MEVALONIC ACID SYNTHESIS

Mevalonic acid (MVA) is the first committed precursor molecule for all isoprenoids (1,3,9). Its synthesis from acetate requires the action of three enzymes: acetoacetyl-CoA thiolase (EC 2.3.1.9), condensing two acetyl-CoA to form acetoacetyl-CoA and HS-CoA; HMG-CoA synthase (EC 4.1.3.5), condensing acetyl-CoA with acetoacetyl-CoA to form HMG-CoA under release of HS-CoA; and HMG-CoA reductase (EC 1.1.1.34), converting HMG-CoA in a two-step reaction by the use of 2 NADPH to form MVA, 2 NADP and free HS-CoA.

HMG-CoA reductase has been widely accepted as playing the regulatory step in isoprenoid synthesis (1,6), although its role in nonmammalian tissues has not been fully defined. Compounds have been synthesized to interfere with early enzymes of the pathway (Fig. 1). SS-polymethylene-bis-methanethiosulfonates ($CH_3SO_2S(CH_2)_nSSO_2CH_3$, n = 6-12) have been shown to inhibit purified mammalian thiolase (24), but were only weakly active as hypocholesterolemic agents (24). This might be explained by the non-limiting activity of thiolase in mammalian tissue. However, in yeast this enzyme seems to be closely regulated (25) and thus might represent a better target for similar compounds.

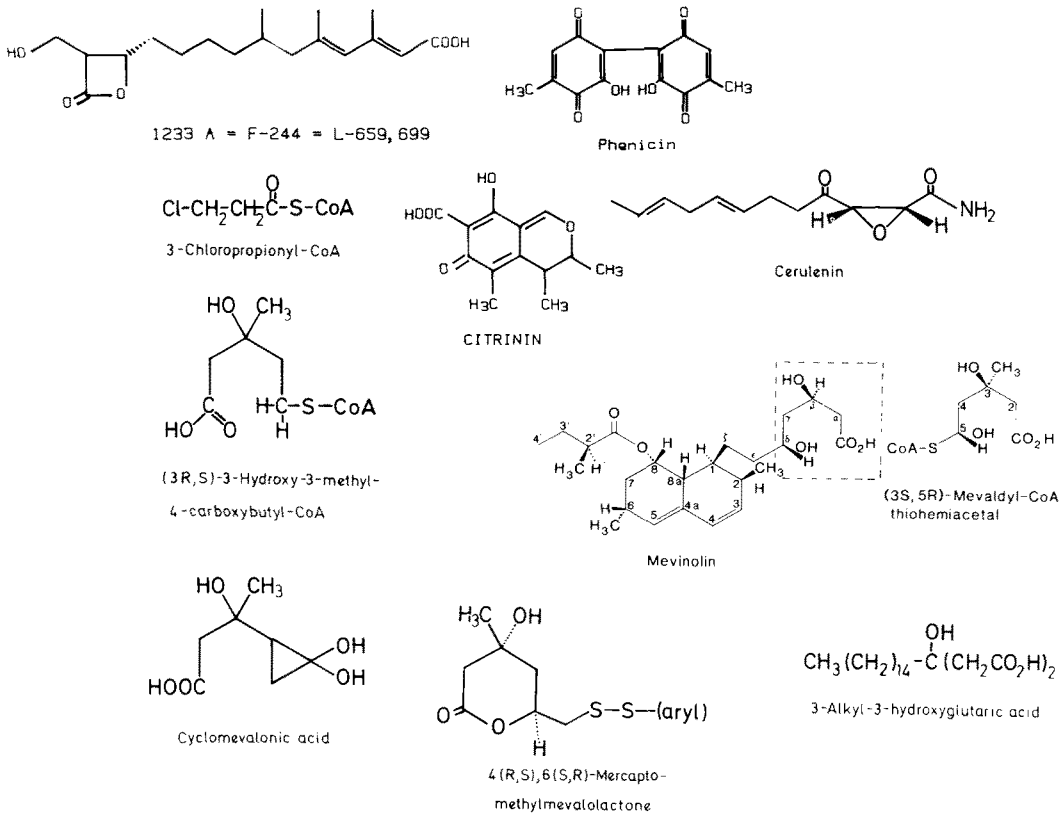

FIG. 1. Selected compounds interfering with the synthesis of mevalonic acid.

Cerulenin, an antibiotic isolated from *Cephalosporum caerulens*, has been reported to inhibit yeast HMG-CoA synthase (26), but its main activity appears to be inhibition of fatty acid and polyketide synthesis. At least in an in vitro system using a heavy-membrane fraction isolated from radish seedlings, we could not detect any inhibition of the enzymic conversion of acetyl-CoA into HMG-CoA (Bach and Weber, unpublished observations). Recent findings (27) also exclude inhibition of the rat liver HMG-CoA synthase.

The thioether analogue of acetoacetyl-CoA, S-(3-oxobutyl-CoA, has been described as interacting with acetoacetyl-CoA utilizing enzymes (28) and was partially converted by avian liver HMG-CoA synthase to (S)-3-hydroxy-3-methyl-4-carboxybutyl-CoA, which by itself proved to be a specific inhibitor of HMG-CoA reductase (29). Another natural antibiotic, citrinin, produced by *Penicillium citrinum* and by several species of *Aspergillus*, was shown to inhibit rat liver and yeast acetoacetyl-CoA thiolase and HMG-CoA reductase (30) in a time-dependent irreversible manner, suggesting binding of the inhibitor to a site distinct from the active center of the reductase molecule (31). However, citrinin has been shown to exert quite different biological activities (32) and is highly toxic (33) as a widespread contaminant in food.

During the course of screening microbes for producing hypocholesterolemic compounds, a fungal fatty acid derivative with a β-lactone moiety, termed F-244 (3,5,7-trimethyl-12-hydroxy-13-hydroxy-methyl-2,4-tetradecadienedioic acid 12,14-lactone) was isolated from strains of *Scopulariopsis* sp. and *Fusarium* sp. and revealed to be a specific inhibitor of rat liver HMG-CoA synthase and of growth of Vero cells (an animal cell line) (27). F-244 had an IC_{50} value for the inhibition of HMG-CoA synthase of 65 μg/L. Independently, the same β-lactone, termed L-659,699, was isolated from *Fusarium* sp. and shown to inhibit a partially purified cytosolic rat liver HMG-CoA synthase with an IC_{50} of 0.12 μM (34). The enzymes HMG-CoA reductase, β-ketoacyl=CoA thiolase, acetoacetyl-CoA thiolase and fatty acid synthetase were not inhibited to any extent by the antibiotic (34). In cultured Hep G2 cells, the compound inhibited ^{14}C-acetate incorporation into sterols with an IC_{50} of 6 μM (34).

Highly interesting is the observation that in cultured Hep G2 cells, the activity of HMG-CoA reductase was induced by the inhibitor in a dose-dependent manner. A 37-fold increase in reductase was observed after a 24-hr incubation with 62 μM L-659,699 (34), thereby pointing to a coordinate control of enzymes involved in mammalian MVA synthesis. The antibiotic, termed 1233A, was originally isolated in 1971 from *Cephalosporum* sp. and was reported as an antibacterial product (35). The potency of the drug as a hypocholesterolemic agent has been demonstrated (34,36). Also, F-244 showed no inhibitory activity (27) towards yeast fatty acid synthetase. Until now, F-244 = 1233A = L-659,699 is the first naturally produced antibiotic that is specifically inhibitory to HMG-CoA synthase and thus might give some support to ascribing a regulatory function to this enzyme (25) in some eukaryotic systems such as yeast.

F-244 is much more active than agents such as 3-chloropropionyl-CoA that cause an active site-directed,

irreversible inhibition of HMG-CoA synthase from avian liver (37,38) but that also react with rat mammary gland fatty acid synthetase (39).

That the enzyme HMG-CoA reductase plays an important ecological role in growth of plant and microbial cells is emphasized through the existence of highly specific antibiotics that bind with and regulate this enzyme. A series of hypocholesterolemic drugs such as compactin and mevinolin and their analogues (1,40) has been isolated from various strains of ascomycetes occurring in the upper rhizosphere. All HMG-CoA reductases tested so far, of eukaryotic as well as prokaryotic origin, are inhibited by these antibiotics, with K_i-values typically being in the range of 10^{-8} to 10^{-9}M. This high affinity of the inhibitors, about three magnitudes higher than towards the natural substrate (S-HMG-CoA), and some structural similarity to (3S, 5R)-mevaldyl-CoA thiohemiacetal, the enzyme-bound intermediate in the two-step reduction of (S)-HMG-CoA to (R)-MVA, led to the conclusion of their representing transient-state analogues. More recent investigations using a yeast enzyme preparation suggest a diffusion-controlled mechanism for the inhibition of HMG-CoA reductase by mevinolin (41).

It is intriguing to postulate two closely neighboring binding sites, one representing the active center, responsible for reacting with the HMG-like moiety of the inhibitor, and the other one binding the less polar (bicyclic) moiety, resulting in some multiplication of the inhibition constants for HMG and HS-CoA, both of which alone are rather weak inhibitors of HMG-CoA reductase (42). Rogers and Rudney (42) observed an apparent irreversibility of the effect of mevinolin or compactin on the immunoinhibition of rat liver HMG-CoA reductase, indicating that these compounds may affect antireductase IgG in a different manner than HS-CoA, HMG-acid or HMG-CoA, perhaps through a tight binding also to other regions of the HMG-CoA reductase molecule, inducing a change in enzyme conformation and causing a blockage of antigenic sites. Alberts et al. (43) already reported that the inhibition type of rat liver reductase by mevinolin slightly depends on the order of mixing enzyme, substrates and inhibitor in the test system.

We and others (1,40) made comparable observations when HMG-CoA reductase preparations from yeast were used. Mevinolin as well as compactin (1,40) are quite potent plant growth regulators. Their low toxicity, their being excellent pharmaceutical tools in the treatment of hypercholesterolemia (Lovastin) as well as their biodegradability through the action of microbial organisms have led to the development of numerous strategies to the organic synthesis of mevinolin and compactin and derivatization (44). The availability of such entities also may lead to their being regarded as parental compounds for newly developed biocides for agricultural purposes.

Recently, phenicin (=phoenicine), a metabolite isolated from cultures of *Penicillium phoeniceum* and *P. rubrum*, was shown to react with solubilized and partially purified rat liver HMG-CoA reductase, apparently through interaction with the sulfhydryl groups of the enzyme molecule (45). Fifty percent inhibition of reductase was measured at 200 μM phenicin, whereas the enzymic synthesis of digitonin-precipitable sterols from ^{14}C-acetate or from ^{14}C-HMG-CoA in a rat liver enzyme system was reduced to 50% at 30 μM (45). A similar discrepancy has been observed for the efficacy of citrinin (30). An artificial substrate analogue of HMG-CoA, S-(4-bromo-2,3-dioxobutyl)-CoA, specifically and irreversibly inactivated both yeast and rat liver HMG-CoA reductase (46). Preincubation with the substrate HMG-CoA, but not with NADPH, protected the enzymes from being inactivated. The inhibition was attributed to reaction with an essential cysteine at the HMG-CoA binding site (46). A number of other compounds were checked for their potency to react with mammalian HMG-CoA reductase and/or to reduce serum cholesterol (47,48). So far, none of those compounds could reach the efficacy and selectivity of compactin/mevinolin and closely related analogues. Investigations with plant material or fungi have not been reported.

INHIBITION OF IPP SYNTHESIS

The synthesis of isopentenyl pyprophosphate (IPP) from MVA requires the action of three enzymes: mevalonate kinase (EC 2.7.4.2), synthesizing 5-phospho mevalonate with ATP as a cosubstrate; 5-phosphomevalonate kinase (EC 2.7.4.2), catalyzing the synthesis of 5-pyrophosphomevalonate; and pyrophosphomevalonate anhydrodecarboxylase (EC.4.1.1.33), decarboxylating and dehydrating 5-pyrophosphomevalonate to form IPP in the presence of ATP.

Most promising analogues, such as 2-fluoromevalonic acid, prepared to block IPP synthesis (Fig. 2) were converted into their pyrophosphates rather than using the corresponding alcohol. Incubation with these inhibitors blocked the decarboxylation step (49), thereby explaining

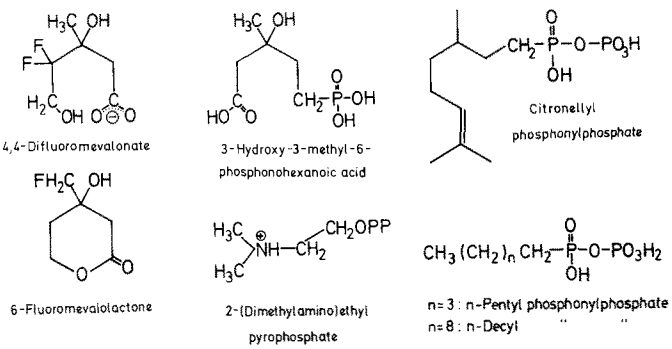

FIG. 2. Inhibitors of IPP formation and of prenyltransferase reactions.

the efficacy of fluorinated MVA in reducing de novo cholesterol synthesis in an isolated perfused rat liver (50).

A strategy based on substituting pyrophosphate groups also has led to the development of inhibitors of prenyltransferases (51,52) and of 5-phosphomevalonate kinase (53). 3-Hydroxy-3-methyl-6-phosphonohexanoic acid, the isosteric analogue of 5-phosphomevalonate, inhibited the latter enzyme in a cell-free system from rat liver with an apparent K_i of 145 μM (53).

INHIBITION OF PRENYLPYROPHOSPHATE FORMATION

Isopentenyl pyrophosphate isomerase (EC 5.3.3.2) catalyzes the interconversion of isopentenyl pyrophosphate (Δ-3-IPP) and dimethylallyl pyrophosphate (DMAPP = Δ-2-IPP), the 5-carbon allylic pyrophosphate subunit ("isoprene") that initiates the formation of all polyisoprenoid molecules by the action of prenyltransferase = dimethylallyltransferase (EC 2.5.1.1.) (54).

Purified yeast isomerase was irreversibly inactivated by 2-(dimethylamino)ethyl pyrophosphate, apparently a transient-state analogue of the intermediate carbonium ion (55) with a remarkably low K_i calculated to be 1.4×10^{-11}M. Substrate analogues without a positively charged nitrogen were relatively poor inhibitors. Also, replacement of the pyrophosphate by phosphate diminished the inhibitory potency minimally 10^4-fold (55). As yet, no in vivo effects of these inhibitors have been reported. Also in this case, it might be suggested to replace the pyrophosphate by phosphonophosphate to prevent hydrolysis by pyrophosphatases and thus to increase stability in biological systems.

The irreversible condensation of an allylic pyrophosphate with IPP and the production of the next higher homologue of the allylic substrate under release of pyrophosphate is catalyzed by prenyltransferase (56,57). There are different prenyltransferases that produce every size of product from C_{10}, C_{15}, C_{20}, up to high molecular weight polymers in plants, gutta percha and rubber. The first enzyme in this series is dimethylallyltransferase (EC 2.5.1.1). This enzyme condenses either a C_5 or a C_{10} allylic pyrophosphate with the homoallylic pyrophosphate to yield geranyl pyrophosphate (C_{10}) and as the ultimate product, farnesyl pyrophosphate, which then serves as a substrate for squalene and sterol synthesis. Quite early (56), inhibition of prenyltransferase in vivo was postulated to be the soundest way of blocking cholesterol manufacture in the human body. Inhibition of this enzyme, in the absence of inhibition of IPP isomerase, would result in the accumulation of DMAPP, a function of the chemical equilibrium of the isomerase that is largely in favor of DMAPP. DMAPP can be hydrolyzed to the free dimethylallyl alcohol, which ultimately can be retroconverted to HMG-CoA via 3,3-dimethylacrylate and *trans*-3-methylglutaconyl-CoA (56). Indeed, this so-called MVA shunt pathway has been demonstrated to operate in mammalian systems (57), in insects (58) and in plants (59). By the action of HMG-CoA lyase (EC 4.1.3.4), HMG-CoA formed in this shunt can be cleaved to be free acetoacetate as the precursor of ketone bodies and acetyl-CoA, thus even regenerating some of the energy invested in the synthesis of DMAPP.

Analogues of pyrophosphates in which carbinol oxygen is replaced by methylene (C-substituted methylphosphonophosphates) were demonstrated to inhibit squalene synthesis in a cell-free system from rat liver and kaurene biosynthesis in the cell-free system from *Ricinus communis*, with the farnesyl and the geranyl analogues having the highest efficacy (51). N-decyl phosphonylphosphate was the most effective inhibitor in a series of alkyl phosphonates and phosphonylphosphates (52). A careful kinetic analysis of the inhibition of purified pig liver prenyltransferase showed that the inhibitor interacted with two forms, or two sites, of the enzyme (52). The finding of two inhibition constants (e.g., 5 μM and 25 μM, respectively) for the decyl phosphonylphosphate indicated its interaction with the free enzyme and the ES_1 complex (S_1=geranyl pyrophosphate, S_2=IPP within the "ordered bi-bi" reaction pathway catalyzed by the liver prenyltransferase). Since both the slopes and vertical intercepts of the Lineweaver-Burk lines were affected when either geranyl pyrophosphate or IPP was the varied substrate, it was concluded that these inhibitors reacted both with the allylic and homoallylic binding sites of the enzyme (52). These observations have been confirmed in a recent study, introducing a novel highly inhibitory analogue, citronellyl phosphonylphosphate (60). As has been pointed out by the latter authors, it appears questionable that such phosphonylphosphates as inhibitors of prenyltransferase are very effective in vivo because a substantial barrier exists against cellular uptake of anionic molecules. Therefore, coupling of the inhibitors was proposed, a strategy that had worked in the case of linking 5-homomevalonophosphonate, an inhibitor of phosphomevalonate kinase (60), through its carboxyl group to the primary hydroxyl group of cholic acid, which then was taken up by hepatocytes (61).

Photoaffinity substrate analogues for prenyltransferases have been demonstrated to be extremely useful tools for identifying and probing the catalytic sites of the enzymes (62, 63). However, the use of such compounds seems to be restricted to in vitro experiments.

Unfortunately, to obtain the desired mode of action on the intact organism, inhibitors or genetic lesions currently designed to block early steps in the sterol pathway are complicated by compartmentation effects, enzymes that are expressed differentially during the growth cycle, and end-products derived from the pathway that are nonsteroidal.

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