Is C-26 hydroxylation an evolutionarily conserved steroid inactivation mechanism?

Steve Meaney

Division of Clinical Chemistry, Karolinska Institutet, Karolinska University Hospital, Huddinge, Sweden

ABSTRACT **Sterols are essential components of virtually all higher eukaryotic organisms, though the exact identity of the dominating sterol varies between species, from the C-27 of cholesterol in vertebrates to the C-28 and C-29 sterols of plants and invertebrates. In addition to their role as structural components of cell membranes these sterols are also converted into a variety of biologically active hormones. This conversion generally involves modifications of the basic structure of the sterol by dealkylation, hydroxylation and/or isomerization. Recent studies have demonstrated that irreversible inactivation of both plant and insect hormones is achieved by a specific C-26 hydroxylation. The concept of sterol deactivation by 26-hydroxylation appears to be an example of an evolutionarily conserved mechanism that has persisted despite the widely varying requirements for sterols in the species where it has been detected.—Meaney, S. Is C-26 hydroxylation an evolutionarily conserved steroid inactivation mechanism? *FASEB J.* 19, 1220–1224 (2005)**

Key Words: · *brassinolide* · *ecdysone* · *27-hydroxycholesterol* · *26-hydroxylase*

Sterols represent a major tool by which eukaryotic cells can refine the properties of their membranes, an ability that is considered an important step in the evolution of higher eukaryotes (1). Although the C-27 sterol cholesterol is the best known of these sterols, other sterols are often quantitatively more important in nonvertebrate species; C-28 and C-29 sterols predominate in plants and invertebrates. The sources of these sterols vary widely, however, with plants and vertebrates capable of de novo synthesis (2) whereas invertebrates such as crustaceans and insects typically require an exogenous source of sterol, at least during certain stages of life (3, 4).

While their importance for the modulation of membrane properties varies between species, sterols invariably act as precursors for different hormones that play essential roles in coordination of development, a key to the survival of any multicellular organism (5). Conversion of a sterol into an active hormone typically requires substantial modification of the parent sterol by processes, including hydroxylation, ω-oxidation, and possible isomerization. This leads to the formation of a steroid that can transduce an appropriate signal by interacting with its cognate receptor, which may be a classical nuclear hormone receptor such as the estrogen receptor or a membrane receptor such as the brassinosteroid receptor (6, 7). As a result of their potent biological activity, it is of paramount importance for an organism to control the active levels of these hormones, and sophisticated mechanisms have evolved to deactivate and subsequently eliminate them (8). Due to the presence of numerous hydroxyl and keto groups, these hormones are suitable for such (phase II type) modifications as glucuronidation and sulfonation. This situation is exemplified by mammalian steroid hormones such as estrogen and testosterone, which are excreted as sulfate and/or glucuronide conjugates.

STEREOCHEMISTRY OF THE C-26 HYDROXYLATION

Sterol 26-hydroxylation corresponds to hydroxylation of one of the two terminal methyl groups of the isopropyl steroid side chain, and may occur in mitochondrial and/or the microsomal fractions of cellular homogenates (9). Due to the nonequivalency of the terminal methyl groups of the sterol side chain, this hydroxylation creates an asymmetric carbon at C-25 and is stereospecific. Thus, hydroxylation at C-26 can lead to the formation of two stereoisomers (25R,26-hydroxycholesterol and 25S,26-hydroxycholesterol, respectively) (10) (see **Fig. 1**). According to present nomenclature, the methyl group that will generate the 25R-isomer when hydroxylated is denoted C-27, and that which will generate the 25R-isomer C-26 (10, 11). At present, information about the natural stereochemistry is only available from certain plants and mammals. In all mammalian systems identified to date, the majority of the 26-hydroxylation leads to the formation of 25R,26-hydroxycholesterol and occurs in the mitochondrial system (9). In contrast, 26-hydroxybrassinbolide is present in plants almost exclusively as the 25S isomer.

Correspondence: Division of Clinical Chemistry (C1-74), Department of Laboratory Medicine, Karolinska Institute, Karolinska University Hospital-Huddinge, S-14186 Stockholm, Sweden. E-mail: steve.meaney@labmed.ki.se

doi: 10.1096/fj.04-3304hyp

0892-6638/05/0019-1220 © FASEB

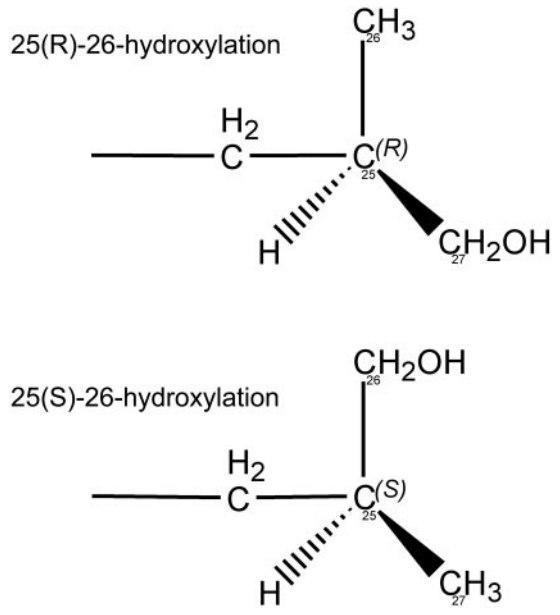

Figure 1. Stereochemistry of C-26 hydroxylation. As a substitution at on of the terminal groups creates an asymmetric carbon at C-25, the C-26 hydroxylation may be stereospecific. Formation of 25R,26-hydroxylated products appears to be a result of a mitochondrial activity whereas 25S,26-hydroxylation appears to be a result of microsomal activity.

However, both stereoisomers are inactive in the rice leaf lamina inclination assay (12). At present the stereochemistry of 26-hydroxylation in species such as *Drosophila* remains to be determined. Due to the lack of information about the stereochemistry at C-25 for all species, and for the purposes of clarity, hydroxylation of either of the terminal methyl groups will be designated as a C-26 hydroxylation.

A POTTED HISTORY OF STEROL SIDE CHAIN HYDROXYLATION

Plants can produce many different brassinosteroids from the parent phytosterol campesterol (5-cholesten-24α-methyl-3β-ol). The most active brassinosteroids are castasterone and brassinolide, the latter considered to represent the prototypical brassinosteroid (13, 14). Brassinolide is synthesized from campesterol via an initial hydrogenation to campestanol, followed by multiple hydroxylations of the side chain and steroid nucleus that yields castasterone and, ultimately, brassinolide. Although it has been understood that 26-hydroxybrassibolide is a significantly less active brassinosteroid (15), it is only in recent years that the evidence has accumulated that castasterone and brassinolide are inactivated by a specific hydroxylation at the C-26 position (see Fig. 3*A*). Neff et al. established that the up-regulation of the cytochrome P450 CYP734A1/BAS-1 (previously classified as CYP72B1) led to a phenotype that essentially mimicked classical brassinosteroid deficiency, with brassinolide levels almost undetectable while 26-hydroxybrassinolide levels increased more than sixfold (16). CYP734A1 was later confirmed as a general brassinosteroid hydroxylase involved in an endogenous steroid inactivation (17). It was recently reported that a highly homologous cytochrome P450, CYP72C1, was also capable of hydroxylating of plant steroid hormones (18). Although the position of the hydroxylation was not determined, the authors speculated that the high homology suggested an overlapping role with CYP734A1, indicating this enzyme may have the capacity to hydroxylate the steroid side chain. To date, the formation of an acidic metabolite of brassinosteroids has not been reported.

A similar situation exists in insects, though with the notable difference that insects cannot cyclize squalene and are sterol auxotrophs: they require small amounts of sterols to survive (3). Insects obtain these sterols from plant and animal tissue, where they convert it into 7-dehydrocholesterol and eventually into ecdysone (see Fig. 3*B*). In recent years many of the steps in this pathway have been characterized at the molecular level, and it is now clear that cytochrome P450s are involved in the C-2, C-20, C-22, and C-25 hydroxylations that lead to the generation of the active ecdysteroids ecdysone and 20-hydroxyecdysone (19–23). Intriguingly, the precursor sterol ecdysone is also capable of acting as a molting hormone, though with less potency than 20-hydroxyecdysone, a situation similar to that of castasterone and brassinolide.

The main route of inactivation of both of these ecdysteroids appears to be conversion into 26-hydroxylated metabolites, and ultimately to the corresponding ecdysonic acids (24). While this has not been detailed at the molecular level, it is highly likely that a mitochondrial cytochrome P450 is largely responsible for this conversion. In an attempt to identify candidate 26-hydroxylases in insects the *D. melanogaster* genome was queried with representative plant and vertebrate 26-hydroxylases (CYP734A1 and CYP27A1, respectively). The sequences identified by these queries were then aligned (using Clustal 1.83) with a selection of known 26-hydroxylases from different species (see **Fig. 2**). Although comparative sequence analysis of P450s must be interpreted with caution, this analysis has revealed several candidate genes that are suitable for subsequent investigation by an expression cloning approach.

The situation in other invertebrates is less well explored. The cholesterol requirements of *C. elegans* are well known, and current evidence points to its role as substrate for hormone formation, although the identity of the cholesterol-derived hormone has remained elusive (25). These investigations are hampered by the vanishingly low concentrations of cholesterol (and the sterol-derived hormone) required by *C. elegans* for normal development. Recently, some clues have emerged about the identity of this steroid. In an elegant study Matyash et al. demonstrated that a sterol-derived hormone (with a polarity significantly greater than that of cholesterol) is a necessary factor for reproduction and prevention of *dauer* larva formation in *C. elegans* (26). Circumstantial evidence was presented in this

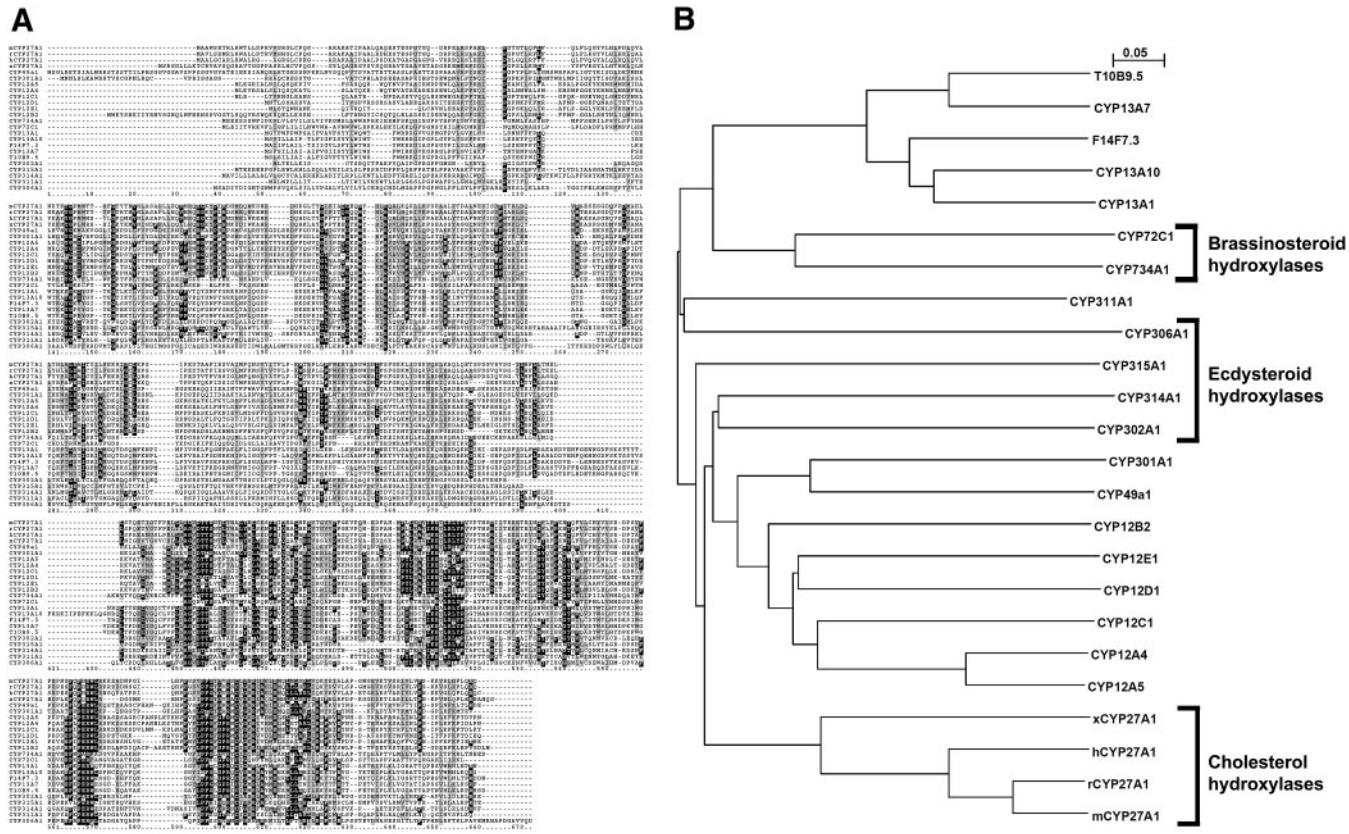

Figure 2. Computational analysis of sterol 26-hydroxylases. The *D. melanogaster* genome was queried using BLAST with the full-length coding sequences of CYP734A1 and CYP27A1. High-quality matches identified by both searches were extracted and aligned using Clustal 1.83. A selection of *C. elegans* (CYP13A10, CYP13A1, F147.3, CYP13A7, and T10B9.5) and vertebrate (human, rat, mouse, and *Xenopus* CYP27A1) P450 sequences were included for comparative purposes. Results of the multiple sequence alignment are shown in Fig. 2*A*. The illustration was generated using Boxshade (http://bioweb.pasteur.fr/seqanal/interfaces/boxshade.html). Black and gray shading indicates identical and similar amino acid residues, respectively. *B)* Phylogenetic analysis of the aligned cytochrome P450s. Known brassinosteroid, ecdysteroid, and cholesterol hydroxylases are indicated.

work suggesting 26-hydroxylation to be an inactivating modification, as 26-hydroxycholesterol was incapable of functionally replacing cholesterol in the experimental systems used in this study (ibid). Moreover the very recent development of a transgenic strain of *C. elegans* capable of converting 7-dehydrocholesterol into cholesterol (27) may facilitate definition of the steroid-derived hormones in nematodes by classical metabolic strategies.

In vertebrates steroid hormones such as testosterone and estrogen are formed after cleavage of the sterol side chain, which precludes further oxidative metabolism of the side chain. However, active side chain hydroxylation of cholesterol occurs in many tissues, most notably in connection with bile acid biosynthesis, leading to the formation of 25R,26-hydroxycholesterol and its corresponding carboxylic acid (see **Fig. 3*C***) (28, 29). This mechanism is an important part of bile acid biosynthesis and may therefore be viewed as an exception to the general contention that this type of hydroxylation is a inactivation step. It is important to realize that this mechanism is of importance in maintaining cholesterol homeostasis and from this point of view represents an "inactivation," though of a structural sterol rather than of a hormone. Hydroxylation of cholesterol renders the sterol metabolically unavailable to a cell and designates it for elimination from the organism (30). As this mechanism may not be absolutely dependent on the availability of apolipoproteins it may represent a more primitive mechanism for elimination of cellular cholesterol. Moreover as 25R,26-hydroxycholesterol may function as a ligand for the LXR family of nuclear receptors, its production may serve to amplify other lipid removal pathways (31).

IS C-26 HYDROXYLATION AN EVOLUTIONARILY CONSERVED STEROID INACTIVATION MECHANISM?

The presence of a capacity to generate C-26 hydroylated sterols is apparently ubiquitous, with 26-hydroxylase typically activities present all hitherto studied phylae of plantae and animalia. The preserved site of modification of the sterols from very different organisms indicates considerable evolutionary pressure to preserve this mechanism, possibly due to an important role in inactivation.

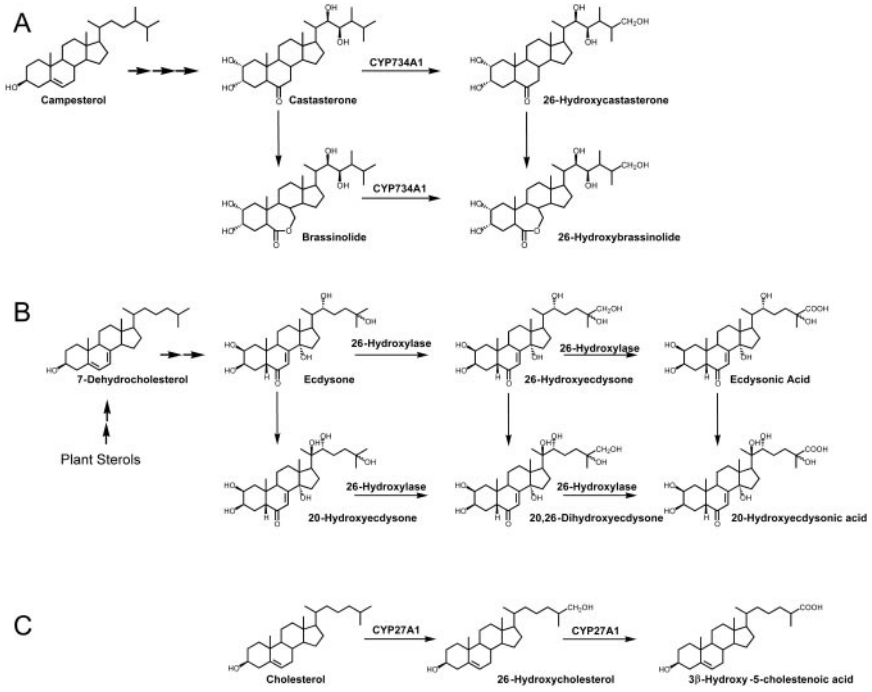

Figure 3. Metabolism of sterols by C-26 hydroxylation in plants (3*A*), insects (3*B*), and mammals (3*C*). As the stereochemistry at C-25 has not been empirically determined for all of the sterols, it is not explicitly defined in these structures. See refs 2, 21, 28 for further details of the pathways leading to the formation of the precursor steroids.

As all of the enzymes capable of this type of hydroxylation identified to date are members of the cytochrome P450 superfamily, it is tempting to speculate that the persistence of C-26 hydroxylation is due to a requirement for a capacity to metabolize potentially dangerous exogenous sterols. For example, it has been suggested that ingestion of material rich in CYP734A1 could lead to a depletion of functional ecdysteroids in phytophagous insects, possibly preventing molting after feeding (16). It has also been suggested that phytohormones may provide a defense against parasitic nematodes (32). An analogous situation could exist in omnivores, where ingestion of sufficient quantities of active steroids could lead to deleterious abnormalities in the absence of a suitable inactivation mechanism. One of the most patent examples of this is the capacity of CYP27A1 to rapidly metabolize 7-oxocholesterol, which is a major dietary oxysterol implicated in atherogeneisis (33–35). A possible implication of these observations is that C-26 hydroxylation may have been coopted by other synthetic pathways (i.e., bile acid biosynthesis) as the capacity for de novo synthesis of cholesterol (and the need to eliminate it) emerged in multicellular organisms. In this regard it is intriguing that C-26 hydroxylated bile alcohols represent the most primitive sterol based intestinal detergents, and that a terminal oxygen function is a necessary modification in all pathways involved in the degradation of cholesterol into bile acids (36, 37).

Further support for possible evolutionary relationships would be provided by identification of invertebrate sterol 26-hydroxylases, followed by detailed examination of substrate specificities from different species, and their potential for multiple hydroxylations of their primary substrates. Finally, it is possible to explore the capacity of hydroxylases from different species to functionally replace one another using genetically modified organisms, for example, the *Cyp27a1* null mouse (38) and other mutants.

Research into the evolutionary history of sterols, once the subject of keen interest (36, 39), has been reinvigorated in recent years. Significant progress has been made in elucidation of the molecular mechanisms of sterol utilization in different species, and this field promises to unearth fascinating discoveries in the coming years. FJ

This work was supported by the Swedish Brain Foundation, Lars Hiertas Minne, Karolinska Institute Research Fund, Loo and Hans Ostermans foundation for geriatric research, Gun and Bertil Stohnes Foundation, and by the Swedish Medical Research Council and the Swedish Heart-Lung Foundation. The author would also like to thank Prof. I. Björkhem for critically reviewing this manuscript and Mr. F. O'Farrell for valuable computational assistance.

REFERENCES

1. Barenholz, Y. (2002) Cholesterol and other membrane active sterols: from membrane evolution to "rafts". *Prog. Lipid Res.* **41,** 1–5
2. Benveniste, P. (2004) Biosynthesis and accumulation of sterols. *Annu. Rev. Plant Biol.* **55,** 429–457
3. Clayton, R. B. (1964) The utilization of sterols by insects. *J. Lipid Res.* **15,** 3–19
4. Kanazawa, A. (2001) Sterols in marine invertebrates. *Fish. Sci.* **67,** 997–1007
5. Thummel, C. S., and Chory, J. (2002) Steroid signaling in plants and insects—common themes, different pathways. *Genes Dev.* **16,** 3113–3129
6. Wang, Z. Y., and He, J. X. (2004) Brassinosteroid signal transduction—choices of signals and receptors. *Trends Plant Sci.* **9,** 91–96
7. Walters, M. R., and Nemere, I. (2004) Receptors for steroid hormones: membrane-associated and nuclear forms. *Cell. Mol. Life Sci.* **61,** 2309–2321

8. You, L. (2004) Steroid hormone biotransformation and xenobiotic induction of hepatic steroid metabolizing enzymes. *Chem. Biol. Interact.* **147,** 233–246
9. Une, M., Kim, H. G., Yoshii, M., Kuramoto, T., and Hoshita, T. (1997) Comparative studies on ω-hydroxylation of 5 β-cholestane-3 α, 7α, 12α-triol in the mitochondrial and microsomal fraction of the liver from several vertebrates. *Steroids* **62,** 458–461
10. Gustafsson, J., and Sjostedt, S. (1978) On the stereospecificity of microsomal "26"-hydroxylation in bile acid biosynthesis. *J. Biol. Chem.* **253,** 199–201
11. Popják, G., Edmond, J., Anet, F. A., and Easton, N. R., Jr. (1977) Carbon-13 NMR studies on cholesterol biosynthesized from [^{13}C]mevalonates. *J. Am. Chem. Soc.* **99,** 931–935
12. Pharis, R. P., Janzen, L., Nakajima, S. K., Zhu, J., and Back, T. G. (2001) Bioactivity of 25-hydroxy-, 26-hydroxy, 25,26-dihydroxy- and 25,26-epoxybrassinolide. *Phytochemistry* **58,** 1043–1047
13. Clouse, S. D. (2003) Recent Advances in brassinosteroid research: from molecular mechanisms to practical applications. *J. Plant Growth Regul.* **22,** 273–275
14. Mussig, C., and Altmann, T. (2001) Brassinosteroid signaling in plants. *Trends Endocrinol. Metab.* **12,** 398–402
15. Seto, H., Fujioka, S., Koshino, H., and Yoshida, S. (1999) Synthesis and biological evaluation of extra-hydroxylated brassinolide analogs. *Tetrahedron* **55,** 8341–8352
16. Neff, M. M., Nguyen, S. M., Malancharuvil, E. J., Fujioka, S., Noguchi, T., Seto, H., Tsubuki, M., Honda, T., Takatsuto, S., Yoshida, S., et al. (1999) BAS1: a gene regulating brassinosteroid levels and light responsiveness in Arabidopsis. *Proc. Natl. Acad. Sci. USA* **96,** 15316–15323
17. Turk, E. M., Fujioka, S., Seto, H., Shimada, Y., Takatsuto, S., Yoshida, S., Denzel, M. A., Torres, Q. I., and Neff, M. M. (2003) CYP72B1 inactivates brassinosteroid hormones: an intersection between photomorphogenesis and plant steroid signal transduction. *Plant Physiol.* **133,** 1643–1653
18. Nakamura, M., Satoh, T., Tanaka, S. I., Mochizuki, N., Yokota, T., and Nagatani, A. (2005) Activation of the cytochrome P450 gene, CYP72C1, reduces the levels of active brassinosteroids in vivo. *J. Exp. Bot.* **56,** 843–840
19. Warren, J. T., Petryk, A., Marques, G., Parvy, J. P., Shinoda, T., Itoyama, K., Kobayashi, J., Jarcho, M., Li, Y., O'Connor, M. B., et al. (2004) Phantom encodes the 25-hydroxylase of Drosophila melanogaster and Bombyx mori: a P450 enzyme critical in ecdysone biosynthesis. *Insect Biochem. Mol. Biol.* **34,** 991–1010
20. Niwa, R., Matsuda, T., Yoshiyama, T., Namiki, T., Mita, K., Fujimoto, Y., and Kataoka, H. (2004) CYP306A1, a cytochrome P450 enzyme, is essential for ecdysteroid biosynthesis in the prothoracic glands of Bombyx and Drosophila. *J. Biol. Chem.* **279,** 35942–35949
21. Gilbert, L. I. (2004) Halloween genes encode P450 enzymes that mediate steroid hormone biosynthesis in Drosophila melanogaster. *Mol. Cell. Endocrinol.* **215,** 1–10
22. Petryk, A., Warren, J. T., Marques, G., Jarcho, M. P., Gilbert, L. I., Kahler, J., Parvy, J. P., Li, Y., Dauphin-Villemant, C., and O'Connor, M. B. (2003) Shade is the Drosophila P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. *Proc. Natl. Acad. Sci. USA* **100,** 13773–13778
23. Warren, J. T., Petryk, A., Marques, G., Jarcho, M., Parvy, J. P., Dauphin-Villemant, C., O'Connor, M. B., and Gilbert, L. I. (2002) Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of Drosophila melanogaster. *Proc. Natl. Acad. Sci. USA* **99,** 11043–11048
24. Koolman, J., Reum, L., and Karlson, P. (1979) 26-Hydroxyecdysone, 20,26-dihydroxyecdysone and inokosterone detected as metabolites of ecdysone in the blowfly Calliphora vicina by radiotracer experiments. *Hoppe Seylers Z. Physiol. Chem.* **360,** 1351–1355
25. Kurzchalia, T. V., and Ward, S. (2003) Why do worms need cholesterol? *Nat. Cell Biol.* **5,** 684–688
26. Matyash, V., Entchev, E. V., Mende, F., Wilsch-Brauninger, M., Thiele, C., Schmidt, A. W., Knolker, H. J., Ward, S., and Kurzchalia, T. V. (2004) Sterol-derived hormone(s) controls entry into diapause in *Caenorhabditis elegans* by consecutive activation of DAF-12 and DAF-16. *PLoS Biol.* **2**(10), e280
27. Lee, E. Y., Shim, Y. H., Chitwood, D. J., Hwang, S. B., Lee, J., and Paik, Y. K. (2005) Cholesterol-producing transgenic *Caenorhabditis elegans* lives longer due to newly acquired enhanced stress resistance. *Biochem. Biophys. Res. Commun.* **328,** 929–936
28. Russell, D. W. (2003) The enzymes, regulation, and genetics of bile acid synthesis. *Annu. Rev. Biochem.* **72,** 137–174
29. Javitt, N. B. (2002) 25R,26-Hydroxycholesterol revisited: synthesis, metabolism, and biologic roles. *J. Lipid Res.* **43,** 665–670
30. Diczfalusy, U., Lund, E., Lütjohann, D., and Björkhem, I. (1996) Novel pathways for elimination of cholesterol by extrahepatic formation of side-chain oxidized oxysterols. *Scand. J. Clin. Lab. Invest. Suppl.* **226,** 9–17
31. Fu, X., Menke, J. G., Chen, Y., Zhou, G., MacNaul, K. L., Wright, S. D., Sparrow, C. P., and Lund, E. G. (2001) 27-hydroxycholesterol is an endogenous ligand for liver X receptor in cholesterol-loaded cells. *J. Biol. Chem.* **276,** 38378–38387
32. Soriano, I. R., Riley, I. T., Potter, M. J., and Bowers, W. S. (2004) Phytoecdysteroids: a novel defense against plant-parasitic nematodes. *J. Chem. Ecol.* **30,** 1885–1899
33. Lyons, M. A., Samman, S., Gatto, L., and Brown, A. J. (1999) Rapid hepatic metabolism of 7-ketocholesterol in vivo: implications for dietary oxysterols. *J. Lipid Res.* **40,** 1846–1857
34. Lyons, M. A., and Brown, A. J. (2001) Metabolism of an oxysterol, 7-ketocholesterol, by sterol 27-hydroxylase in HepG2 cells. *Lipids* **36,** 701–711
35. Lyons, M. A., and Brown, A. J. (2001) 7-Ketocholesterol delivered to mice in chylomicron remnant-like particles is rapidly metabolized, excreted and does not accumulate in aorta. *Biochim. Biophys. Acta* **1530,** 209–218
36. Haslewood, G. A. (1967) Bile salt evolution. *J. Lipid Res.* **8,** 535–550
37. Björkhem, I. (1992) Mechanism of degradation of the steroid side chain in the formation of bile acids. *J. Lipid Res.* **33,** 455–471
38. Rosen, H., Reshef, A., Maeda, N., Lippoldt, A., Shpizen, S., Triger, L., Eggertsen, G., Björkhem, I., and Leitersdorf, E. (1998) Markedly reduced bile acid synthesis but maintained levels of cholesterol and vitamin D metabolites in mice with disrupted sterol 27-hydroxylase gene. *J. Biol. Chem.* **273,** 14805–14812
39. Bergman, W. (1958) Evolutionary aspects of the sterols. In *Cholesterol: Chemistry, Biochemistry, and Pathology* (Cook, R. P., ed) pp. 435-445, Academic Press, New York

Received for publication December 9, 2004.
Accepted for publication April 4, 2005.