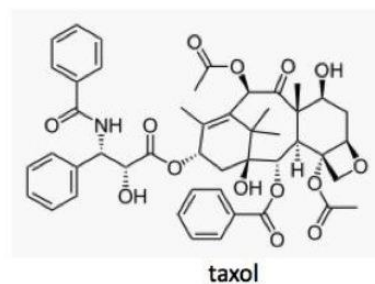
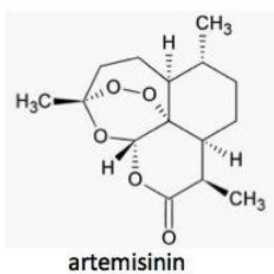
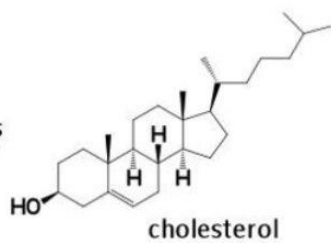
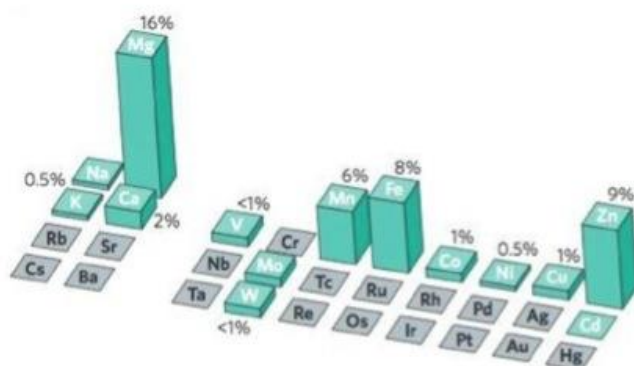


5.08 Exam #3

Name: _____



What do these natural products share in common?



Point Distribution:

Problem 1 (20 points)

1.

2.

3.

Problem 2 (30 points)

1.

2.

3.

4.

5.

6.

Problem 3 (50 points)

1.

2.

3.

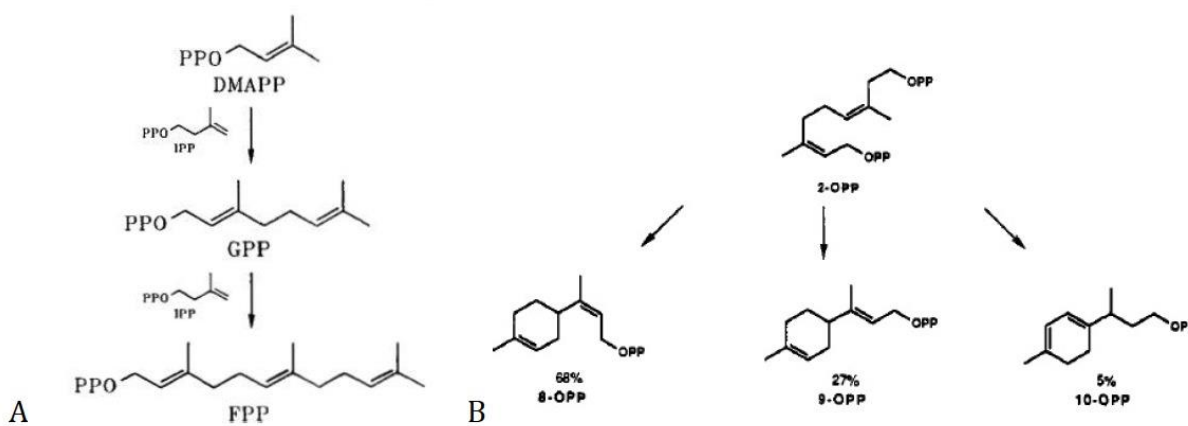
4.

5.

6.

Total _____

1. (20 points) Farnesyl pyrophosphate (FPP) synthase has been extensively studied and catalyzes the reaction shown in Figure 1A. FPP synthase can also use 2-OPP as an alternative substrate (Figure 1B).



© American Chemical Society. Davisson, V.J., T.R. Neal, and C.D. Poulter. "Farnesyl-diphosphate synthase. Catalysis of an intramolecular prenyl transfer with bisubstrate analogs." *J. Am. Chem. Soc.*, 1993, 115 (4), pp 1235–45. All rights reserved. This content is excluded from our Creative Commons license. For more information, see <https://ocw.mit.edu/help/faq-fair-use>.

Figure 1. FPP synthase catalyzes the reaction in panel A of 2 IPP and 1 DMAPP to make FPP via the GPP intermediate and B. the reaction of FPP synthase with 2-OPP to form the three products shown.

Questions (20 points)

- (5 points) Propose a detailed mechanism for formation of geranylIPP from IPP and DMAPP. Draw out each step and show the proposed intermediate(s).

2. **(10 points)** Another way to probe the presence of an intermediate you proposed in question 1 is to use substrate analogs. 2-OPP in Figure 1B was synthesized and studied with FPP synthase to gain mechanistic insight about the FPP synthase catalyzed reaction. The products of the reaction are also shown in Figure 1B.

a. Propose a mechanism by which these products might be generated

b. Given the types of reactions given in class that are prototypes for the chemistry you have described in question 1, what other product might be observed if you looked harder (had higher sensitivity methods of detection)?

3. (5 points) The terpenome is composed of > 70,000 natural products. What is a unique feature of enzymes involved in making isoprenes and terpenes that distinguish these enzymes with most enzymatic reactions that you have learned about? (Hint, think about FPP)

2. (30 points) This question was taken from J Lipid Res 49, 399 2008. HepG2 cells (liver cells) were grown in delipidated serum for up to 24 h and then the cells were transferred to fresh delipidated serum with 1 μ M lovastatin; 50 μ M sodium mevalonate (this concentration supplies the limited amount of this metabolite required for non-isoprenoid compounds essential for cell survival) in the presence or absence of sterols for the times indicated in Figure 2. The cell lysates were subjected to immunoblots with polyclonal antibodies against PCSK9 (proprotein convertase subtilisin/kexin type 9), low density lipoprotein receptor (LDLR), and sterol-regulatory element binding protein 2 (SREBP-2). The GAPDH protein was used as a loading control. P and C for PCSK9 are its proprotein (intracellular) and cleaved forms (extracellular).

A similar set of experiments in which subsequent to lovastatin induction, increasing concentrations of mevalonate were examined are shown in Figure 3.

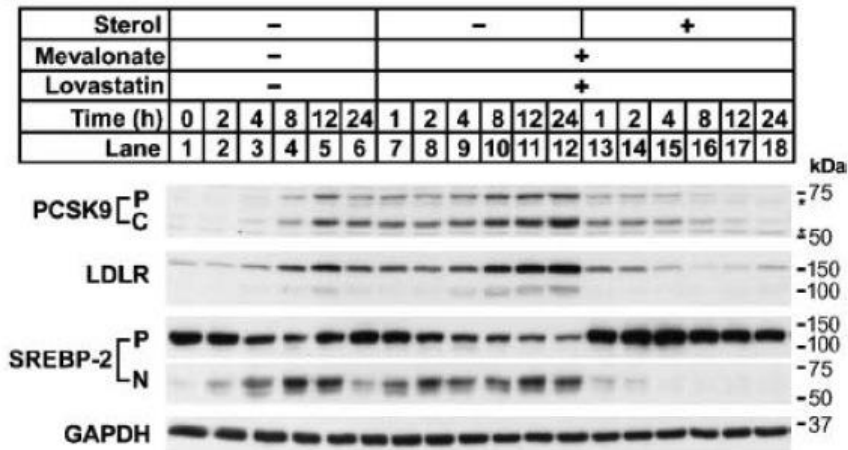
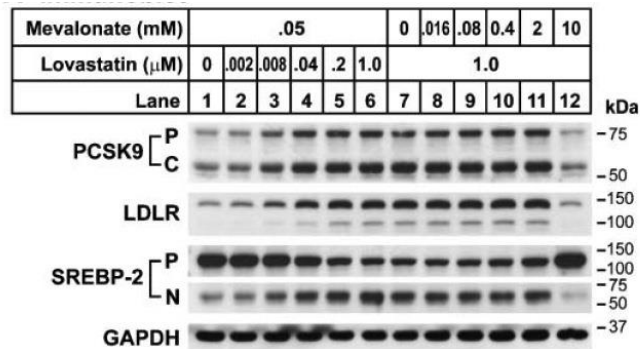


Figure 2. Time-course expression of PCSK9 in HepG2 cells after depletion or supplementation of sterols. HepG2 cells were grown and then switched at time 0 to delipidated serum for the indicated times with the indicated components (lanes 1-6); similar growth conditions in delipidated serum with 1 μ M lovastatin and 50 μ M doisum mevalonate in the absence (lanes 7-12) or the presence (lanes 13-18) of 1 μ □/mL cholesterol. After the indicated incubation times the cells were harvested, lysed and the whole cell lysates were subjected to immunoblot analysis with antibodies to PCSK9 or LDLR or SREBP-2. GAPDH was used as a control.

© American Society for Biochemistry and Molecular Biology. Jeong, H.J., H.S. Lee, et al. "[Sterol-dependent regulation of proprotein convertase subtilisin/kexin type 9 expression by sterol-regulatory element binding protein-2.](#)" *The Journal of Lipid Research*, 49 (2008):399-409. All rights reserved. This content is excluded from our Creative Commons license. For more information, see <https://ocw.mit.edu/help/faq-fair-use>.

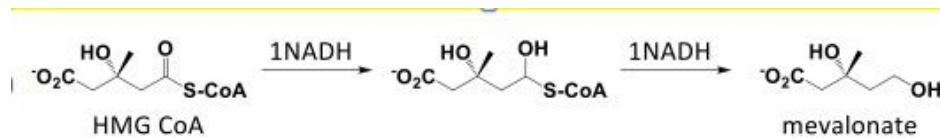


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Figure 3 Induction of PCSK9 by lovastatin and the effects of different concentrations of mevalonate. The growth conditions and analysis are similar to those described in Figure 1.

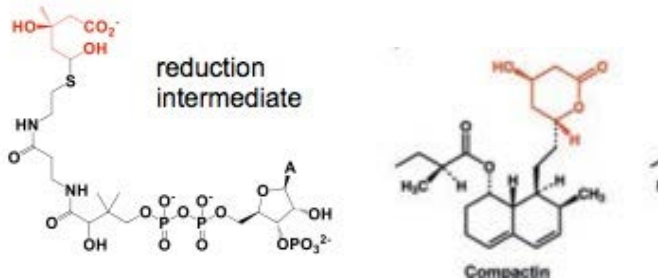
Questions (30 points)

- (3 points) In the cholesterol biosynthetic pathway, what is the rate-limiting step and what is the reaction catalyzed by this step (shown reactants and products)?



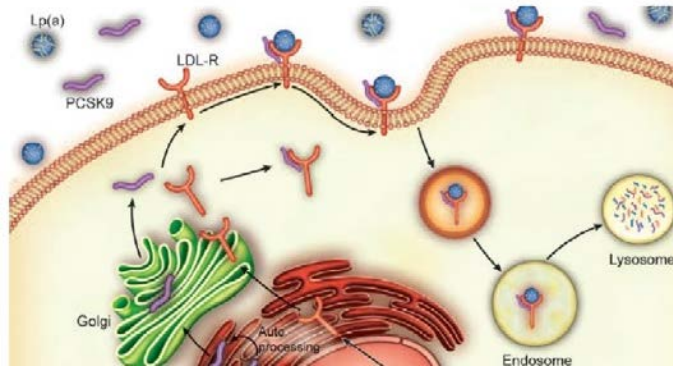
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- (2 points) What is the purpose of addition of lovastatin in the experiments described above and what are the expectations given its metabolic target?



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- (5 points) From recitation 8, provide the model for the role of PCSK9 in cholesterol homeostasis and why this protein is of interest clinically for lowering cholesterol levels.



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4. **(4 points)** In Figure 2, describe what you see from the data.

5. **(6 points)** (In Figure 3, describe what you see from the data. Given the proposed function of PCSK9 described in recitation 8 and your answer to question 3, rationalize why the parallel observations for LDLR and PCSK9 in Figure 3 are a paradox.

6. **(10 points)** In both Figures 2 and 3, the antibodies to SREBP-2 reveal two proteins labeled P and N.
- a. Why are there two apparent forms of SREBP-2? SREBP resides in the ER membrane when sterol levels are high.
 - b. What is the function of SREBP-2 (in a sentence describe the BIG picture)?
 - c. Propose a cartoon model (like those described in class/notes) for the mechanism of active SREBP-2 production.

3. **(50 points)** Hepcidin is a peptide hormone that functions as the master regulator of systemic iron that maintains the levels of the plasma concentration of iron from 10 to 30 μM , despite fluctuations of iron in the diet. Studies predominantly on macrophages, involved in iron recycling from red blood cells, suggest that hepcidin functions by binding to Fpn extracellularly and targeting it for ubiquitination intracellularly which leads to Fpn degradation by the proteasome. The role of hepcidin has been postulated to be similar for iron uptake from the diet.

Very recently the function of hepcidin was examined in enterocytes (**Figure 4**) using cell culture (Caco cells) and in mouse slices from the duodenum (small intestine). Growth conditions similar to those used in the macrophage studies were chosen after much experimentation. The following experiments (**Figure 5, 6 and 7**) were carried out in cell culture with similar results in mouse duodenum slices. In the first set of experiments, optimized conditions (details not given), 0.2 μM hepcidin was incubated with Caco cells for 2 h and then western analysis was performed on crude membranes subsequent to SDS PAGE analysis and electroblotting, using antibodies specific for DMT1 and Fpn1. The results are shown in the western blot in **Figure 5A**, which was then quantitated as shown in **Figure 5B**.

The results shown in **Figure 5** suggested a second experiment in which Caco cells were incubated for one hour in the presence or absence of 50 μM MG-132 (a proteasome inhibitor) and then with or without hepcidin for 1 or 2 hours. Western analysis was performed on crude membranes as described above with antibodies specific for DMT1 and Fpn1. The results of the western analysis is shown in **Figure 6A** and the quantitative analysis is shown in **Figure 6B**.

The results in **Figure 6** suggested one last set of experiments shown in Figure 7. In this case the experiment is a little more complex in that the Caco cells were transfected with [IRE+]-DMT1-eGFP construct and studied under the similar conditions described in **Figures 5 and 6**. In this case the growth was carried out in the absence of presence of hepcidin over a 60 min time course. One additional experiment contained both hepcidin and PYR-41, an inhibitor of E1, the Ub activating enzyme. In this case the cells were lysed and immunoprecipitation carried out with GFP antibodies and then analyzed by western blotting using anti-DMT1 antibodies and antiubiquitin antibodies. The results are shown in Figure 7.

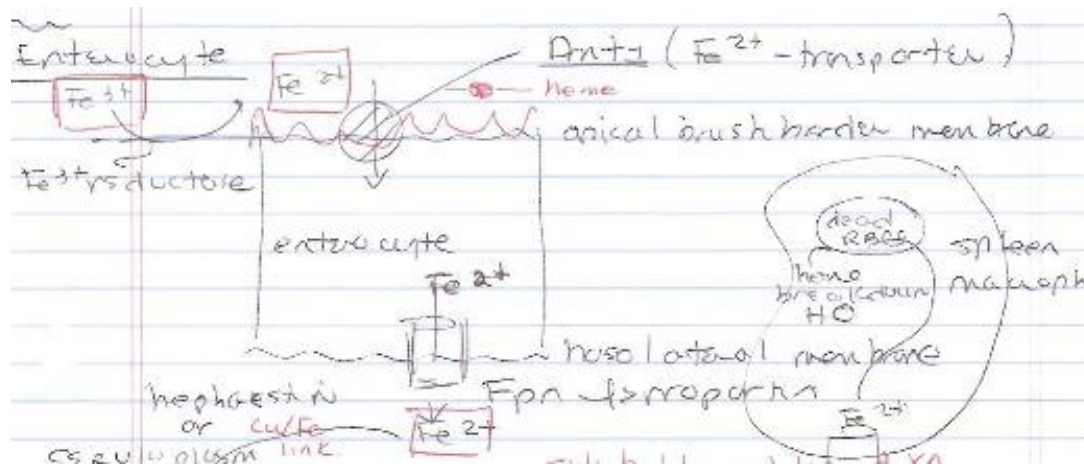
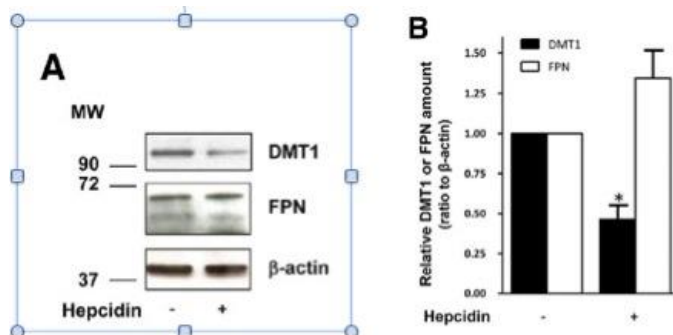
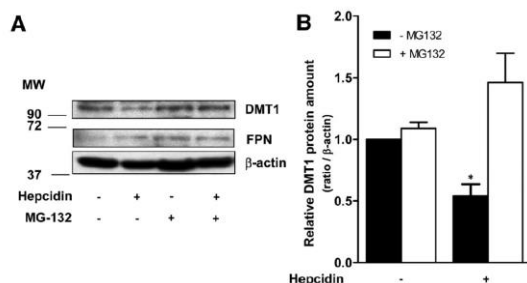


Figure 4. An enterocyte found in the small intestine. The circle and rectangle are proteins of interest in iron homeostasis. Note that diet iron enters enterocytes from the apical brush border membrane.



© The AGA Institute. Carole Brasse-Lagnel, C., K. Zoubida, et al. "[Intestinal DMT1 Cotransporter Is Down-regulated by Hepcidin via Proteasome Internalization and Degradation](#)." *Gastroenterology* 2011, 140(4):1261-1271. All rights reserved. This content is excluded from our Creative Commons license. For more information, see <https://ocw.mit.edu/help/faq-fair-use>.

Figure 5. The effect of hepcidin on DMT1 and FPN protein in Caco cells. A. The cells were incubated for 2 hours with 0.2 μ M hepcidin. At a defined time based on extensive experimentation (not described), crude membranes of the cells were analyzed by Western blotting using rabbit anti-mouse FPN antibody or with rabbit anti-rat DMT1 antibody. B. Quantitative analysis of the data in A.



© The AGA Institute. Carole Brasse-Lagnel, C., K. Zoubida, et al. "[Intestinal DMT1 Cotransporter Is Down-regulated by Hepcidin via Proteasome Internalization and Degradation](#)." *Gastroenterology* 2011, 140(4):1261-1271. All rights reserved. This content is excluded from our Creative Commons license. For more information, see <https://ocw.mit.edu/help/faq-fair-use>.

Figure 6. Western analysis of Caco cells incubated with in the presence or absence of hepcidin and the presence or absence of MG-132 a proteasome inhibitor. A the western blot using antibodies of DMT1 or FPN with actin as the loading standard. B. quantitative assessment of the results in A shown for DMT1. **Note you were not given an “quantitative” analysis of the western data for Fpn. The eyeball method is challenging.**

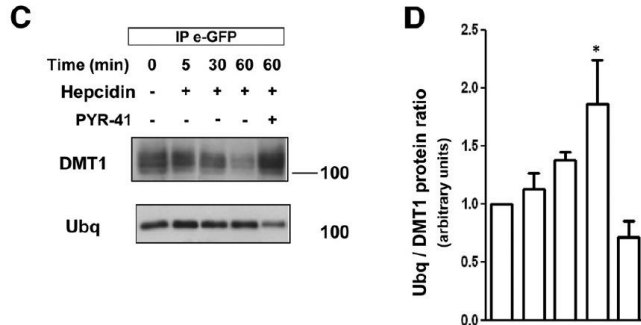


Figure 7. C. IP with E-GFP antibodies to GFP-DMT1 fusion protein under the conditions specified at the top of the Figure and over a 60 min period in the presence or absence of hepcidin. In one case PYR-41, an E1 activating enzyme inhibitor, was also present. The crude lysates were analyzed by western blotting with DMT1 antibodies or Ub antibodies. **D.** quantitation of part **C**.

© The AGA Institute. Carole Brasse-Lagnel, C., K. Zoubida, et al. "[Intestinal DMT1 Cotransporter Is Down-regulated by Hepcidin via Proteasome Internalization and Degradation.](#)" *Gastroenterology* 2011, 140(4):1261-1271. All rights reserved. This content is excluded from our Creative Commons license. For more information, see <https://ocw.mit.edu/help/faq-fair-use>.

Questions (50 points) :

- (5 points)** In **Figure 4** you are given a cartoon of an enterocyte with a circular and rectangular protein. In addition you are given that Fe^{3+} is available from the diet. Write the name and function of the two proteins. What is missing in this diagram to allow Fe^{3+} to be taken into the enterocyte (assign proteins and draw the missing reaction on Figure 4)?
- (5 points)** Describe what you see in **Figure 5**. What is unusual about these observations relative to the proposed universal model by which hepcidin functions derived from similar experiments on macrophages?
- (5 points)** Describe what you see in **Figure 6** and explain how these results provide a possible explanation for the results in **Figure 5**.
- (10 points)** Describe what you see in **Figure 7** in the first four lanes in **C** and **D**. Describe with a cartoon how these observations can further account for the observations in **Figures 5 and 6**.

5. **(5 points)**. In the fifth lane in **Figure 7** in the presence of PYR-41 a potent inhibitor of E1, explain the observed result in terms of cascade of events that appear to account for all the data in this figure.
6. **(20 points)** Both DMT1 and Fpn play important role in homeostasis in are regulated at the translation level. Given the functions for these proteins draw the picture of the mRNA for each protein labeling the 5' and 3' ends. Show how you would expect each protein to be regulated at low iron and at high iron.

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Spring 2016

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