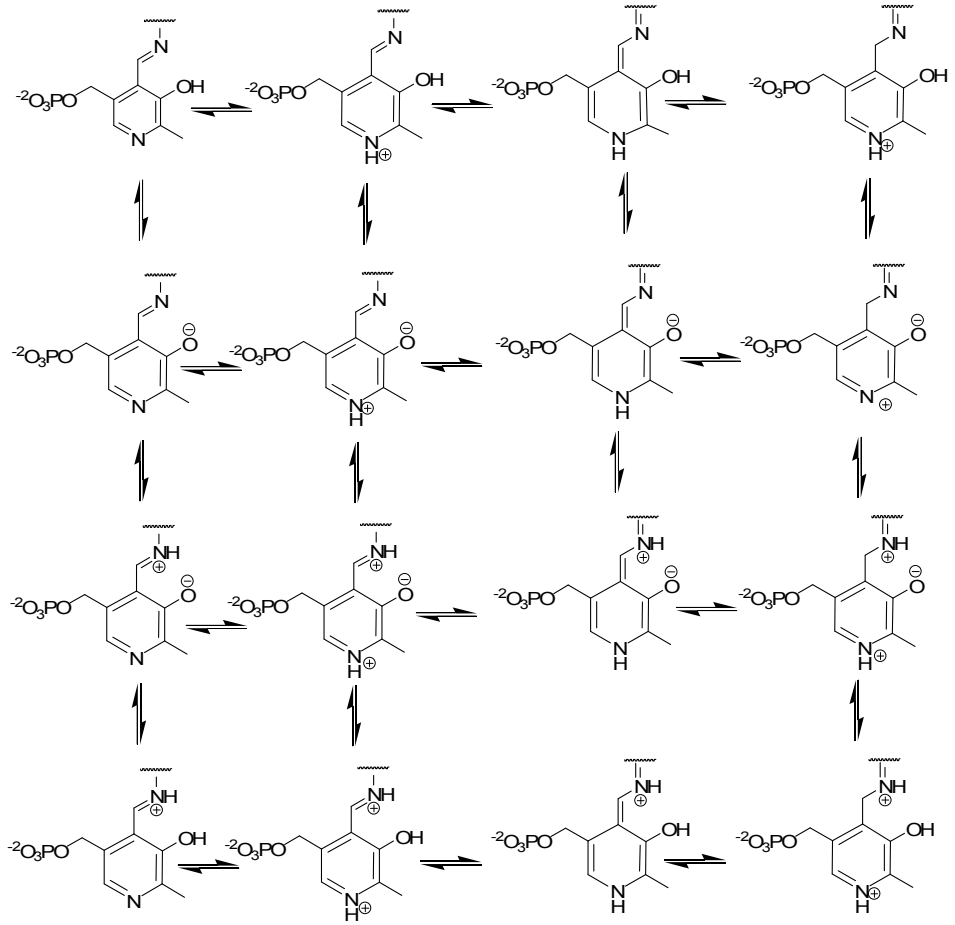
Chem 313 Problem Set #8, **2014**

Before you start PS:

a) Reactions involving three molecules in concerted fashion are unusual and entropically unfavorable in solution under standard condition. Unless it is stated that the reaction happens in an enzyme or in heterogeneous phases, avoid drawing a tri-molecular process which involves the collision of three molecules leading to a concerted reaction.

b) In the problem set answer keys, different protonation states, resonance forms and tautomers of PLP are used (see scheme below).



Which one is most likely to occur depends on the enzyme and the type of reaction. In the literature, the pyridine nitrogen N-1 has traditionally been assumed to be protonated in enzyme active sites, with the protonated pyridine ring providing resonance stabilization of carbanionic intermediates and good electron sink. This assumption is certainly correct for some PLP enzymes, but the structures of other active sites are incompatible with protonation of N-1, and, consequently, these enzymes are expected to use PLP in the N-1 unprotonated form. However, it is unlikely that you have both the protonated N-1 pyridine and the protonated imine/enamine with the neutral O-3' phenol in the majority of enzyme active sites. It is possible that, in many enzyme, the O-3' phenol of the PLP is involved in the tautomerization of the imine/enamine. In addition, the protonation status of the

phosphate could vary. However, to avoid confusion, stick to what you've seen in class.

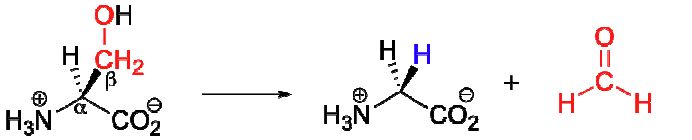
c) Unless you need to practice in more details for the exam, or otherwise stated, it is OK in this problem set to begin with the substrate-PLP adduct, and end with the product-PLP adduct. In other words, you do not need to show the Schiff base being formed and later hydrolyzed.

Questions for PS are starting now:

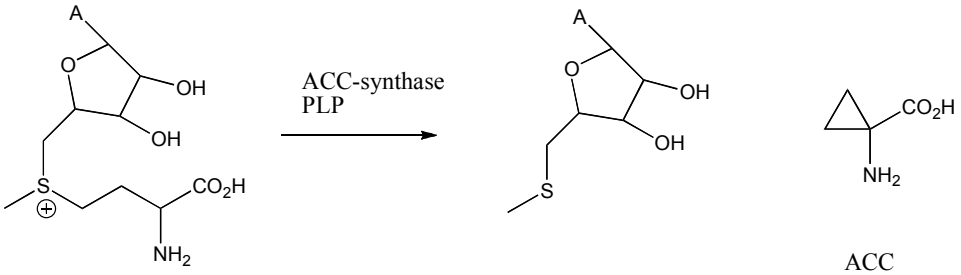
1. When PLP is protonated at the aromatic ring (pyridinyl), what is the effect (increase or decrease) on the aldehyde electrophilicity and on the pKa of the 3-hydroxyl compare to the non protonated PLP?

2. The net reaction of α-ketoglutamate transaminase is shown below. Fill in the unknown products **A** and **B**.

3. Propose a mechanism (only main steps) for the retro-aldol reaction where the C_α-C_β bond of the amino acid side chain is cleaved using PLP.

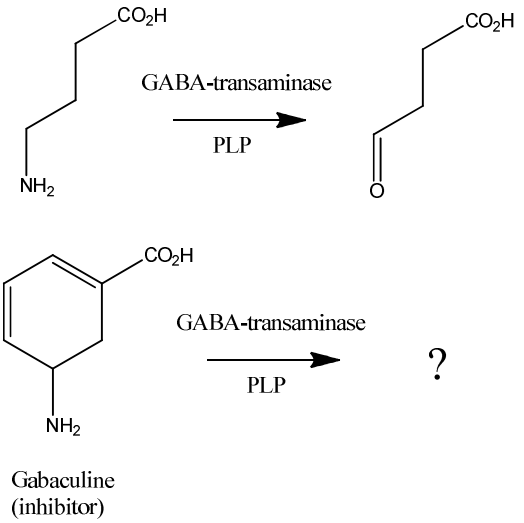


4. The formation of amino-cycloproyl carboxylate (ACC) is an important step in plant metabolism, leading to the formation of ethylene. A PLP-dependent enzyme –ACC-synthase - performs this reaction with S-adenosyl methionine (SAM) as the substrate. Propose a mechanism for this reaction.



SAM ACC

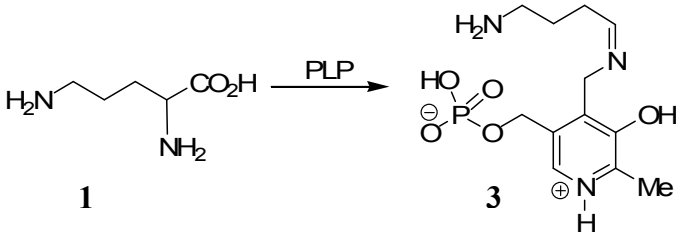
5. Gabaculine is an irreversible inhibitor of γ-aminobutyrate transaminase (reaction shown below). The gabaculine reaction forms an irreversible PLP-gabaculine conjugate, thus killing the enzyme. Propose a mechanism of how gabaculine inhibits this enzyme.



6. Many pyrrolidine (five-membered cyclic amine) alkaloids, such as nicotine, are made from the amino acid ornithine (**1**). For example, the biosynthesis of hygrine (**2**) starts with ornithine reacting with PLP.

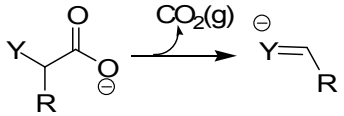
1 **2**

a) Give the mechanism of the ornithine (**1**) transformation into the intermediate pyridoxal imine (**3**).

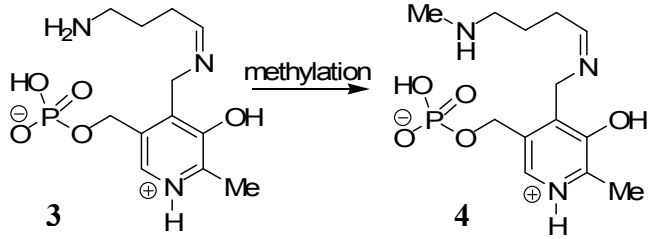


1 **3**

Hint: A decarboxylation is involved (Y = electron withdrawing group able to delocalized, stabilized the negative charge,could be conjugated to a π-system…)

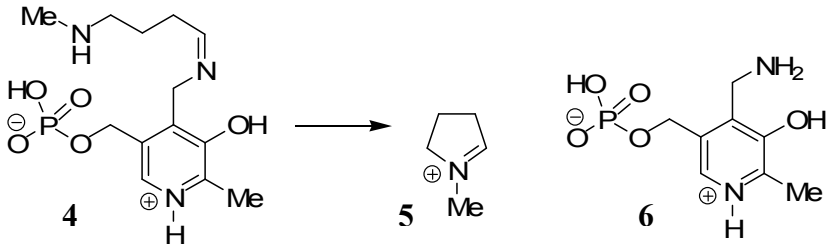


b) The intermediate pyridoxal imine (**3**) is methylated to yield the derivative of *N*-methyl- pyridoxal imine (**4**).



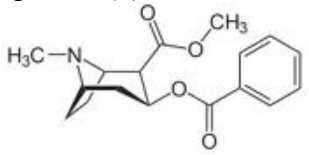
3 **4**

Then, under protons transfer and an entropically favored reaction, the 1-methyl-1-pyrrolidine iminium (**5**) and the pyridoxamine (**6**) are made. Give the mechanism.



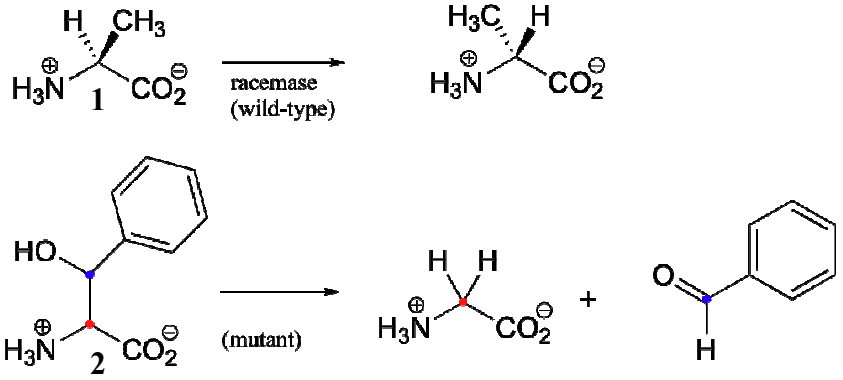
4 **5** **6**

The pyridoxamine (**6**) is recycled back to PLP with the help of an aminotransferease as seen in class. The 1-methyl-1-pyrrolidine iminium (**5**) is a strong electrophile and will be further modified by other pathways to yield hygrine (**2**). Hygrine will undergo further biotransformation to yield many different pyrrolidine-based alkaloids such as the well known benzoylmethyl ecgonine (**7**).

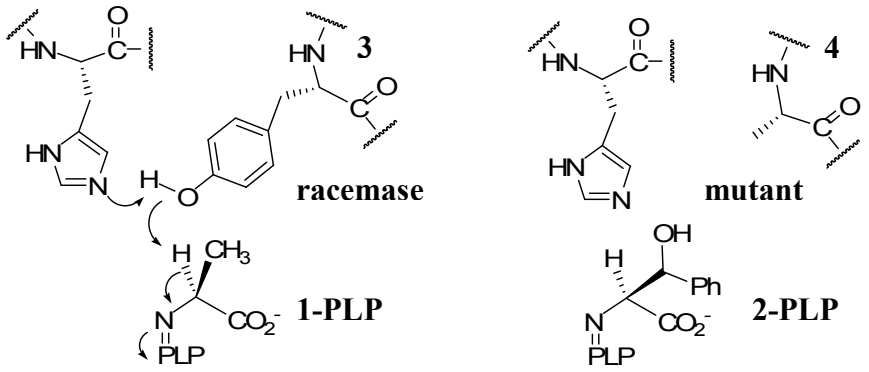


7

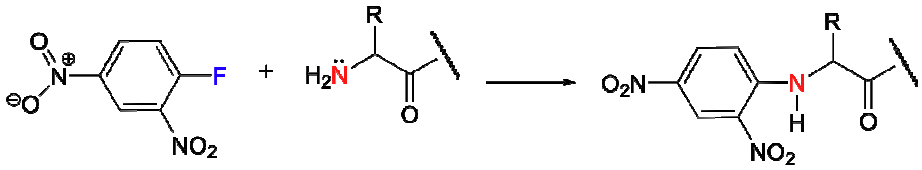
7. We have seen how PLP-dependent enzymes catalyze a group of reaction types - racemizations, retroaldols, transamininations, and eliminations - which, despite their apparent diversity, are all characterized by a critical carbanion intermediate that is stabilized by the electron sink of the PLP coenzyme. Given the similarities in the chemistry, it would be reasonable to propose that the active site architecture of these enzymes might also be quite close. This idea was nicely illustrated by an experiment in which site-directed mutagenesis on a single active site amino acid of PLP-dependent alanine racemase was sufficient to turn it into another reaction type enzyme when provided with a suitable alternate substrate (*J. Am. Chem. Soc*. **2003**, 125, 10158).



The catalytic base that abstracts the α-proton in the alanine racemase reaction is a tyrosine, assisted by a nearby histidine (see figure below). When researchers mutated the tyrosine (**3**) to an alanine (**4**) in the enzyme, and substituted beta-hydroxytyrosine (**2** or **2-PLP** when coupled to PLP) for the alanine (**1** or **1-PLP** when coupled to PLP) substrate, another reaction was catalyzed with remarkable efficiency. Propose a mechanism.

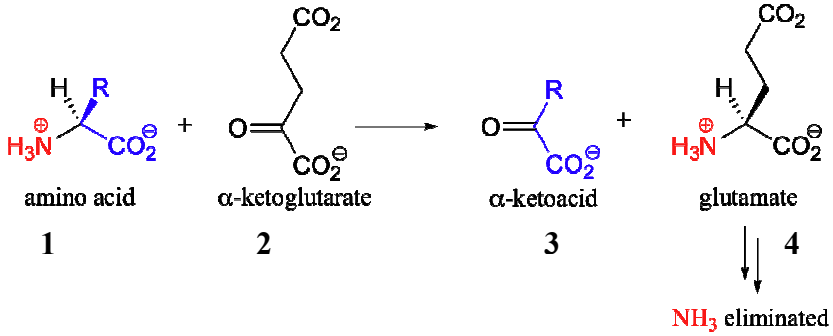


8. Not only PLP is used as electron sink. This concept is also use in org. chem. such as the nitro group conjugated to an aromatic system. As one example of a nucleophilic aromatic substitution that is useful in the laboratory, 2,4-dinitro fluorobenzene can be used to specifically alkylate the N-terminal amino acid in a protein:



This reaction was developed by Frederick Sanger, a pioneer in protein chemistry. **a**) Draw a complete mechanism for the reaction above, clearly showing how the nitro groups stabilize the negative charge on the intermediate; **b**) Use a resonance argument to explain how the presence of a second nitro group serves to further activate the aromatic ring in the nucleophilic aromatic substitution; **c**) Why do you think a fluorine substituent is used on the aromatic ring instead of a better leaving group such as bromine or chlorine (think about rate limiting step which involved a reaction which a high energy barrier, such as breaking bonds that are highly stable)?

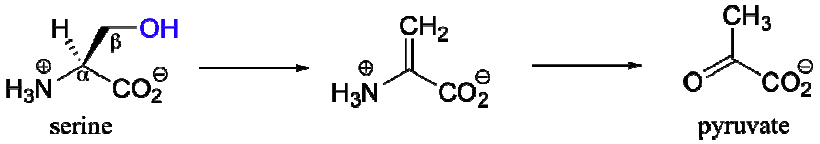
9. One of the most important steps in the degradation of amino acids is elimination of the amino nitrogen atom in the form of urea, which is excreted in the urine. While the metabolic details of this 'urea cycle' are outside of the scope of this class, what is important to understand is that nitrogen atoms from many of the amino acids must be shuttled first to glutamate before being processed for elimination:



The reaction in which the nitrogen group from an amino acid (**1**) is transferred to alpha-ketoglutarate (**2**) is accomplished by PLP-dependent enzymes called transaminases. Once again, the first step is abstraction of the alpha-proton from the

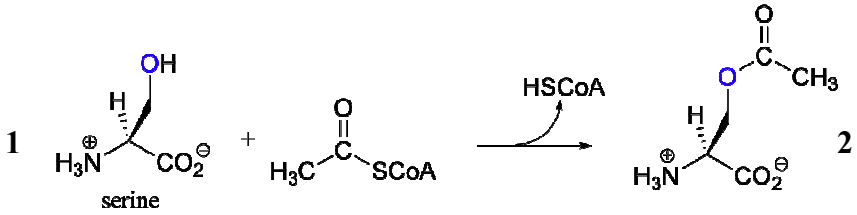
PLP-amino acid adduct. **a**) Propose a mechanism of the first sequences which use only (**1**) and the PLP to release (**3**) and install the amine on the PLP now called pyridoxamine phospate (PMP); **b**) The coenzyme (PMP), which now carries an amine, next transfers the amine group to alpha-ketoglutarate (**2**) (to form glutamate (**4**)) through an exact reversal of the whole process we have just seen. Show a complete, step-by-step mechanism for the second half of the transaminase reaction (transfer of the amine group from PLP to α-ketoglutarate to form glutamate).

10. Additional reaction types in the PLP toolbox are β-eliminations and β-substitutions. **a**) Propose a mechanism of the serine dehydratase catalyzing a β-elimination of the hydroxyl group of serine, leading eventually to pyruvate.



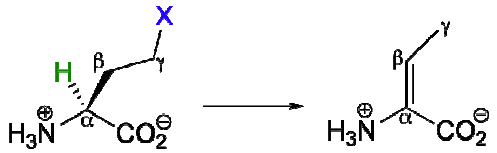
P.**S**.: β-elimination is a chemical reaction in which atoms or groups are lost from adjacent atoms, resulting in a new π-bond: A-B-C-D ---> A + B=C + D. One of atoms lost is usually (but not always) a proton. The new π-bond is usually (but not always) formed between two carbon atoms.

b) In many bacteria, the synthesis of cysteine from serine (**1**) relies upon a PLP-dependent β-substitution step. A β-substitution is simply β-elimination followed directly by the reverse reaction (Michael addition) with a different nucleophile. In this pathway, serine (**1**) is first acetylated by acetyl-CoA (an acyl transfer reaction) to give a β-acetylated serine (**2**).

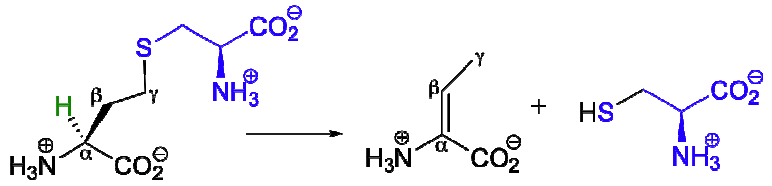


Describes the PLP mechanism involved in the synthesis of cysteine from serine (**1**). Also, what is the need of acetylation of serine in the first step?

11. The electron sink capability of PLP allows some enzymes to catalyze eliminations at the gamma (γ) carbon of an amino acid side chain, rather than at the beta-carbon:

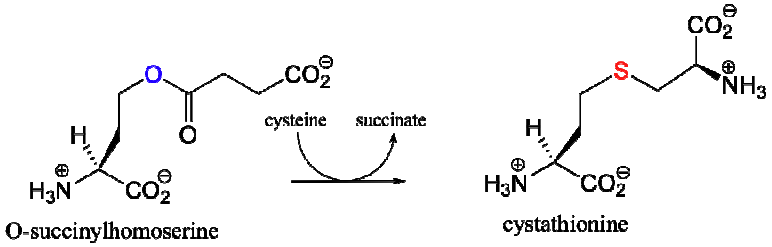


The secret to understanding the mechanism of a gamma-elimination is that PLP acts as an electron sink *twice* - it absorbs the excess electron density from not one but two proton abstractions. **a**) Propose a mechanism of the cystathionine gamma-lyase reaction, which is part of the methionine degradation pathway.



cystathionine 2-aminocrotonate cysteine

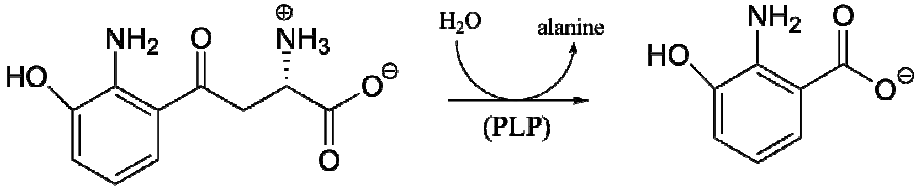
b) Using what you have learned from (**a**), propose a mechanism of the synthesis of methionine, cystathionine (the starting compound in the previous gamma-elimination!) obtained from O-succinyl homoserine and cysteine in a PLP-dependent gamma-substitution.



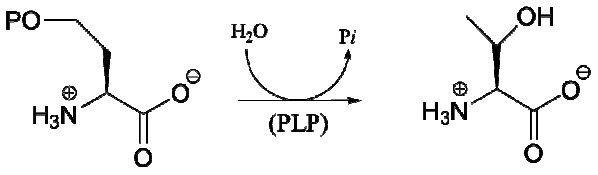
O-succinylhomoserine cystathionine

12. All of the following reactions are PLP-dependent. Draw mechanisms, showing the 'electron sink' action of PLP. In each case, begin the substrate-PLP adduct, and end with the product-PLP adduct (in other words, you do not need to show the Schiff base being formed and later hydrolyzed).

a)

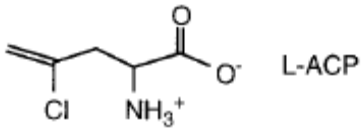


b)

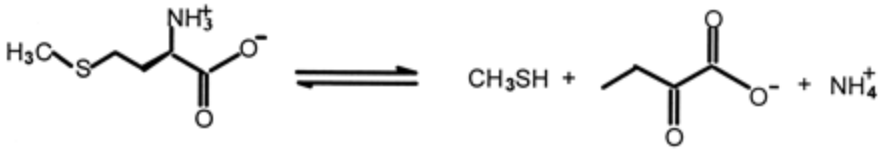


phosphohomoserine threonine

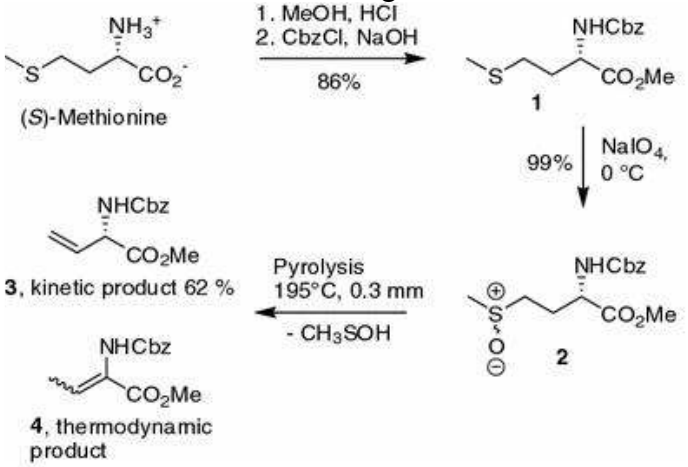
13. L-2-amino-4-chloro-4-pentenoic acid (L-ACP) is a natural product isolated from fruit bodies of Amanita mushroom. It inhibits the growth of bacteria.



Extensive studies revealed that the target of this inhibitor is L-methionine gamma-lyase which catalyzes the conversion of L-Met to α-ketobutyrate, methanethiol and ammonia (Eq.1). This lyase is dependant on pyridoxal 5'-phosphate (PLP).



The following results were obtained from various experiments on this lyase:
i) This enzyme catalyzes the rapid exchange in D_2O (D = deuterium) of both the α- and β-hydrogens of straight chain L-amino acids such Ala and α-aminobutyrate that are <u>not susceptible to elimination</u>.
ii) This enzyme catalyzes the conversion of vinyl glycine to α-ketobutyrate. Protected version of vinyl glycine can be made in solution corresponding to the kinetic product **3** as described in the following scheme:



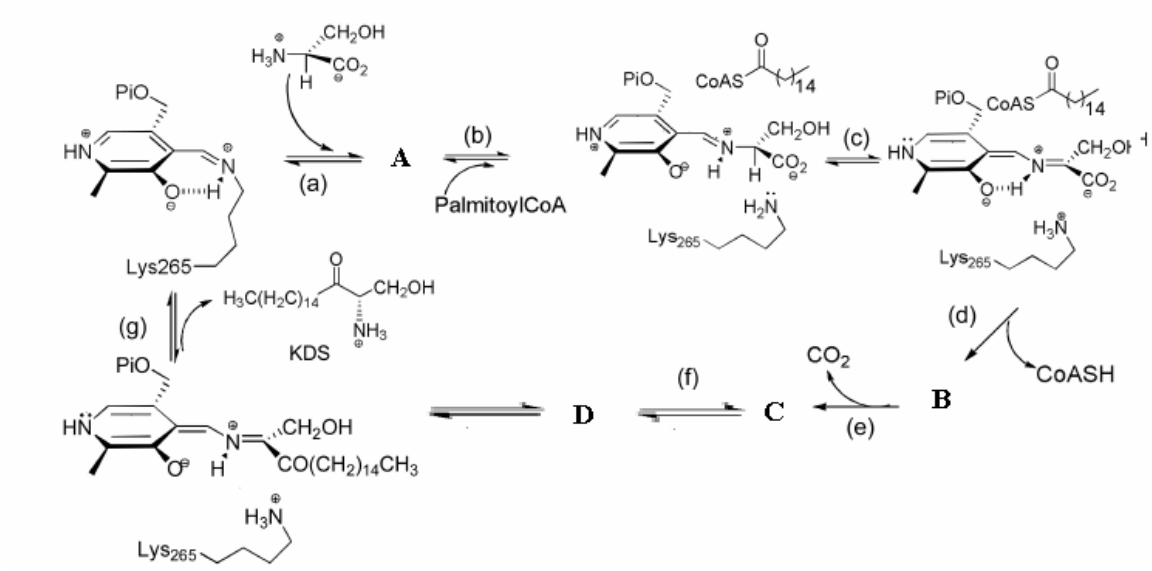
Scheme taken from *Amino Acids* **2010**, 39, 443.

iii) However, vinyl glycine can also inhibit the enzyme after being installed on PLP by making a covalent link to a catalytic residue on the enzyme involved in PLP activity. Many inhibitor of PLP dependent enzyme are based on vinyl glycine such as vigabatrin against GABA aminotransferase. You can look up the structure of vigabatrin.
iv) The lyase is irreversibly inhibited in a time dependent fashion by L-ACP. When [^{14}C]-ACP is incubated with the enzyme, a peak of radioactivity co-elutes with the enzyme through a Sephadex G-25 column (column gel that separates proteins from small molecules). This reaction occurs with 4 moles of [^{14}C]-ACP per tetramer of the enzyme.
Questions: From the information above, propose a mechanism on how does L-methionine gamma-lyase catalyze the conversion of L-Met to alpha-ketobutyrate, methanethiol and ammonia (Eq.1). Explain in mechanistic terms the results obtained in experiments (i), (ii) and (iii for vinyl glycine).

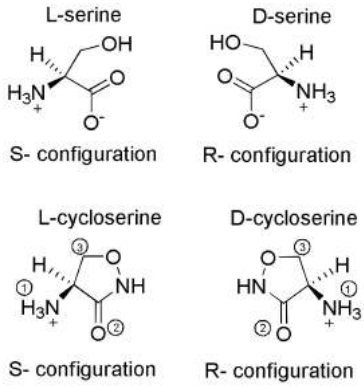
14. Sphingolipids are a large family of bioactive molecules that are found in all eukaryotic and some prokaryotic membranes. It follows that pharmaceutical intervention that regulates the sphingolipid metabolic pathway could help to combat pathological processes such as carcinogenesis, atherosclerosis and Parkinson's disease. The de novo biosynthetic pathway for sphingolipids varies from one

organism to another but the first and rate-limiting step is common to all: condensation of L-serine with palmitoyl-CoA to form 3-ketodihydrosphingosine. This step is catalysed by the pyridoxal 5′-phosphate (PLP)-dependent enzyme serine palmitoyltransferase (SPT), a member of the α-oxoamine synthase (AOS) subfamily as described partially in the scheme below.

Question: Complete the scheme below and give mechanism involved in each step.



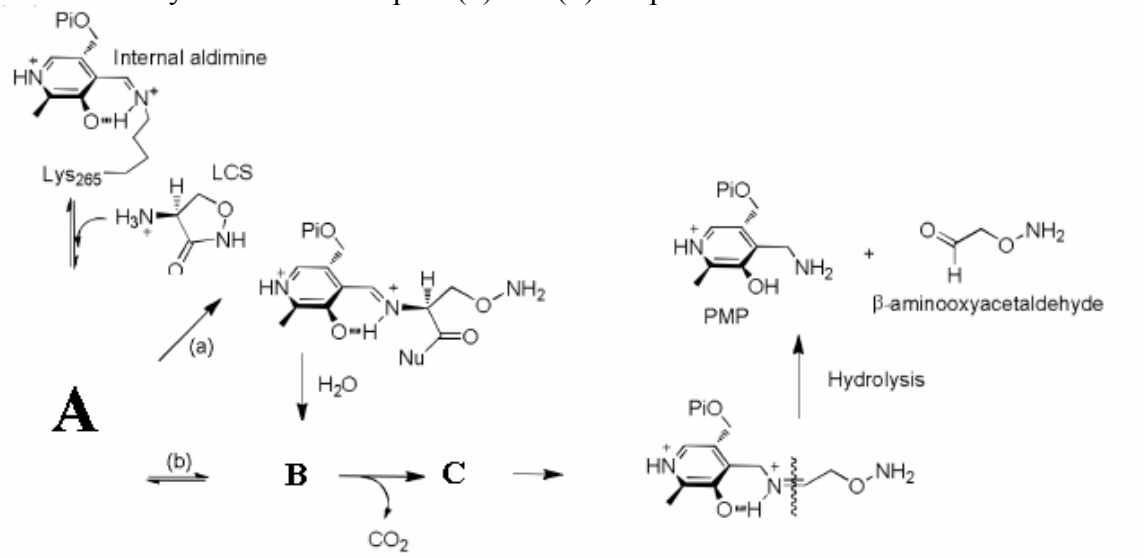
15. The pyridoxal 5′-phosphate (PLP)-dependent enzyme serine palmitoyltransferase (SPT) was also used to study the mechanism of action of the inhibitor cycloserine (CS, 4-amino-3-isoxazolidone). CS is a cyclic amino acid mimic that is known to inhibit many essential pyridoxal 5′-phosphate (PLP)-dependent enzymes. Two CS enantiomers are known; D-cycloserine (DCS, also known as Seromycin), is a natural product that is used to treat resistant Mycobacterium tuberculosis infections as well as neurological disorders since it is a potent NMDA receptor agonist, and L-cycloserine (LCS), is a synthetic enantiomer whose usefulness as a drug has been hampered by its inherent toxicity arising through inhibition of sphingolipid metabolism. Unlike many irreversible inhibitors that inactivate their protein targets by covalent modification, CS renders its targets inactive by forming a stable adduct with the essential PLP cofactor. Both enantiomers of cycloserine can be thought of as cyclic analogues of serine and/or alanine.



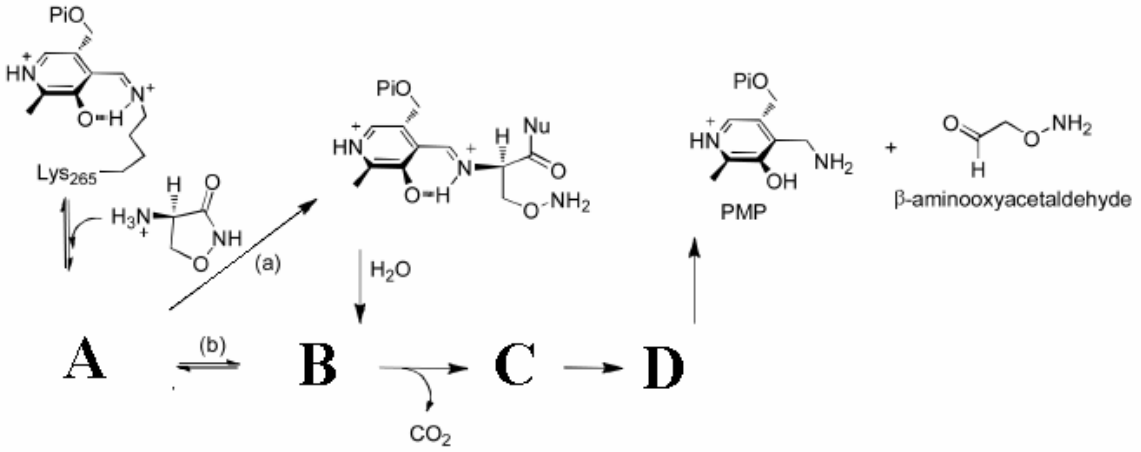
It was postulated that both LCS and DCS inactivate SPT by transamination to form a free pyridoxamine 5′-phosphate (PMP) and β-aminooxyacetaldehyde that remain bound at the active site. We suggest this occurs by ring opening of the cycloserine ring followed by decarboxylation.

Question: Complete the scheme below and give mechanism involved in each step.

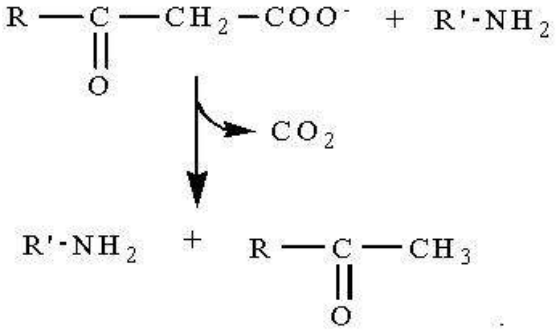
i) Inhibition of SPT by LCS. Here two path (**a**) and (**b**) are possible from intermediate **A**.



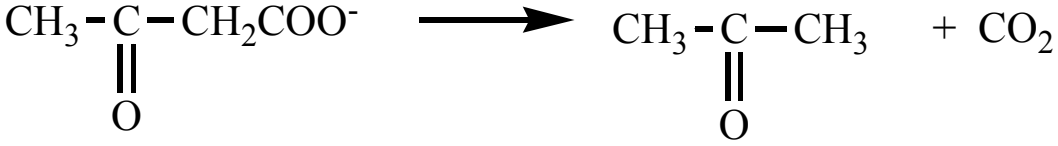
ii) Inhibition of SPT by DSC.



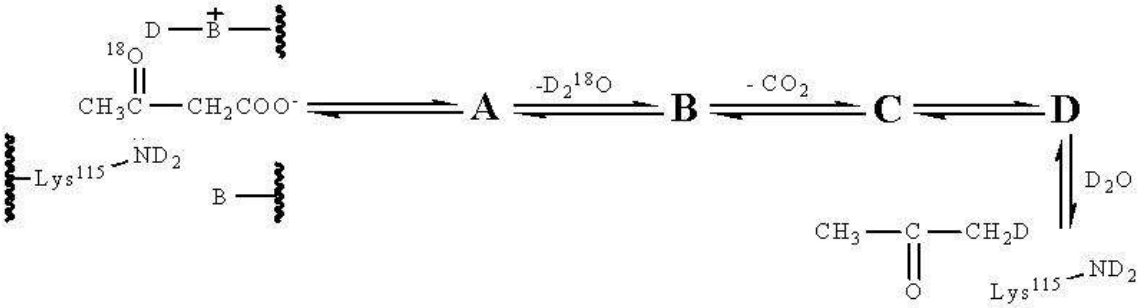
16. Back to basic by describing the amine-catalyzed decarboxylation of β-keto acids without PLP.



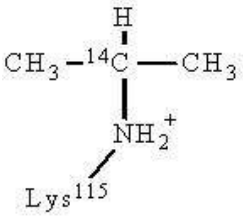
17. PLP is not always needed for enzyme such as the reaction catalyzed by Acetoacetate Decarboxylase.



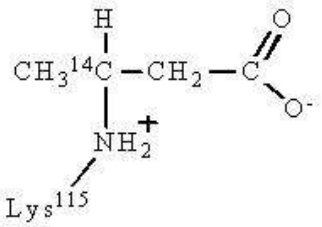
a) The fate of the ketone oxygen and alpha protons in the reaction catalyzed by acetoacetate decarboxylase were studied using ^{18}O labeled acetone and D_2O (D is deuterium, ^{2}H). Knowing that the enzyme is using its Lys^{115} to make an imine intermediate, explain the mechanism using the isotopic $D_2{}^{18}O$. You can use one or more equivalent of general base (RB:) and acid (RBD^+) for deuterium cation (D^+) transfer.



b) Using same substrate and enzyme as in question (**a**), if you wait for CO_2(g) bubbling from the enzyme activity before adding $NaBH_4$, then hydrolyze all amide bonds (protein hydrolysis) with acid and heat, you isolate compound **8.9**. However, if you add first $NaBH_4$, then no CO_2(g) bubbling is observed and you isolate compound **8.10** after amide bond hydrolysis. Explain how these results further proof that imine formation is a key intermediate.



8.9



8.10