INHIBITORS

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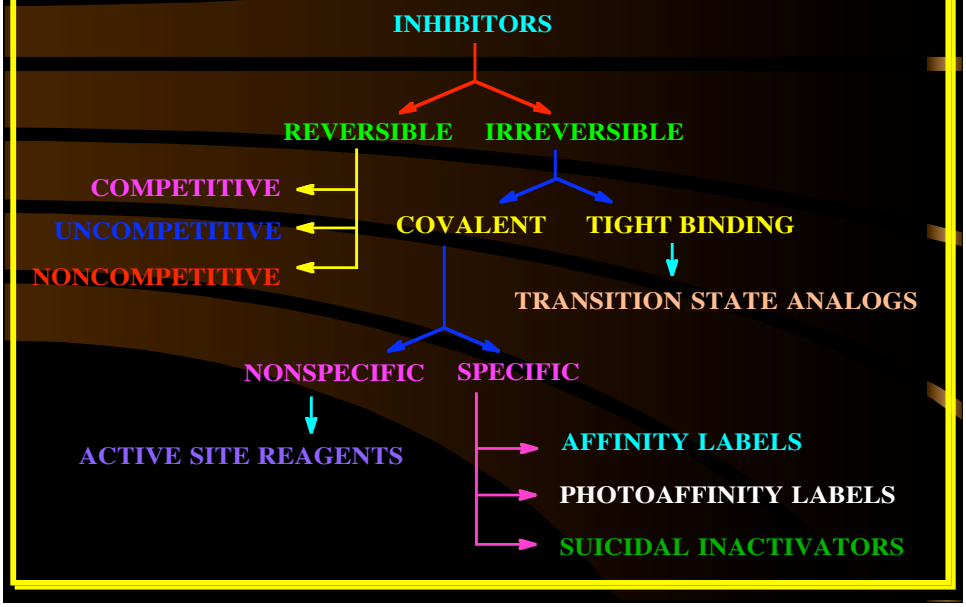
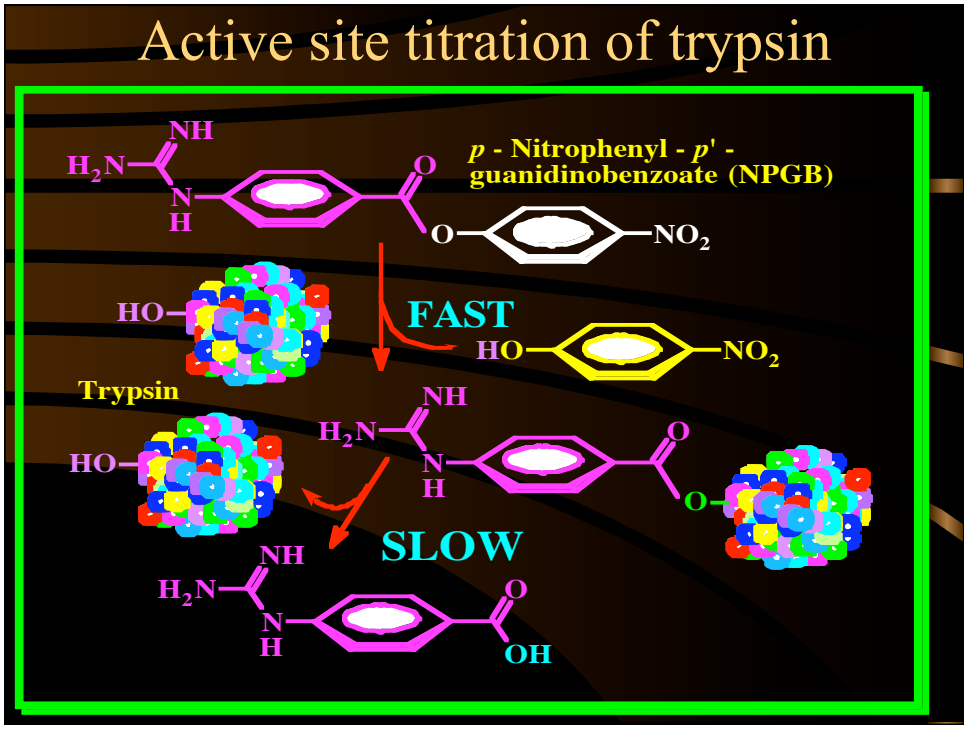
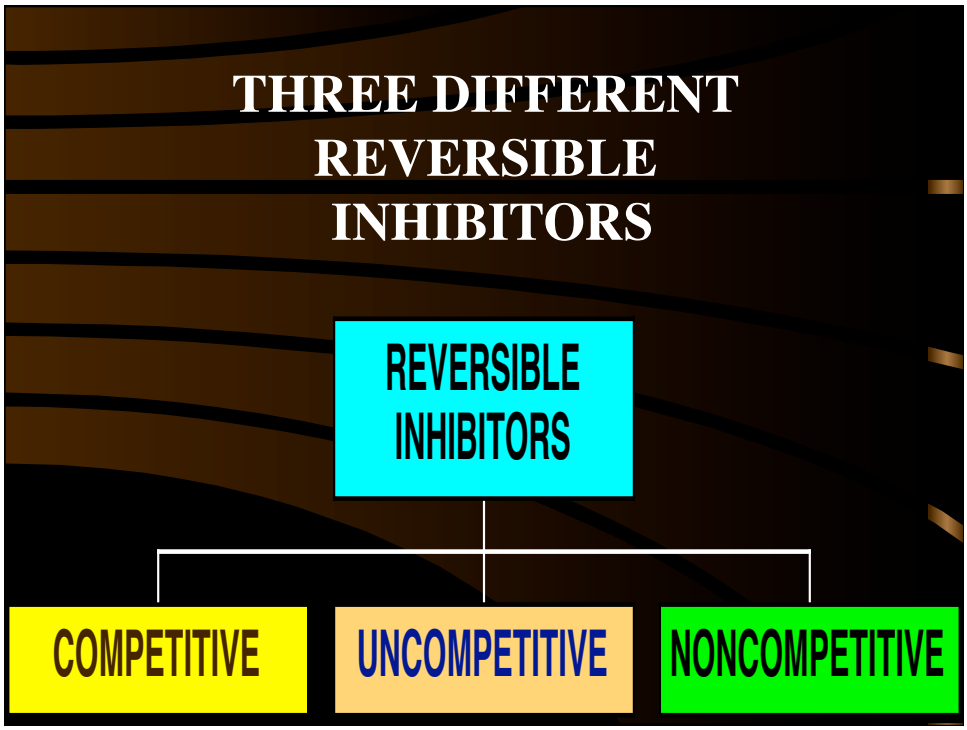
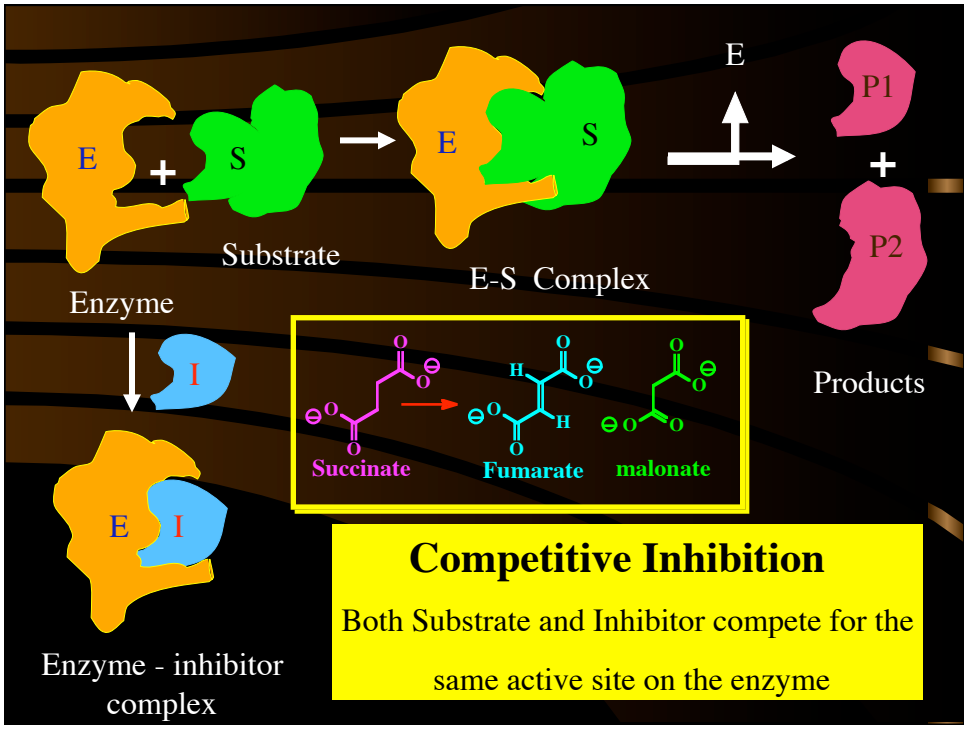
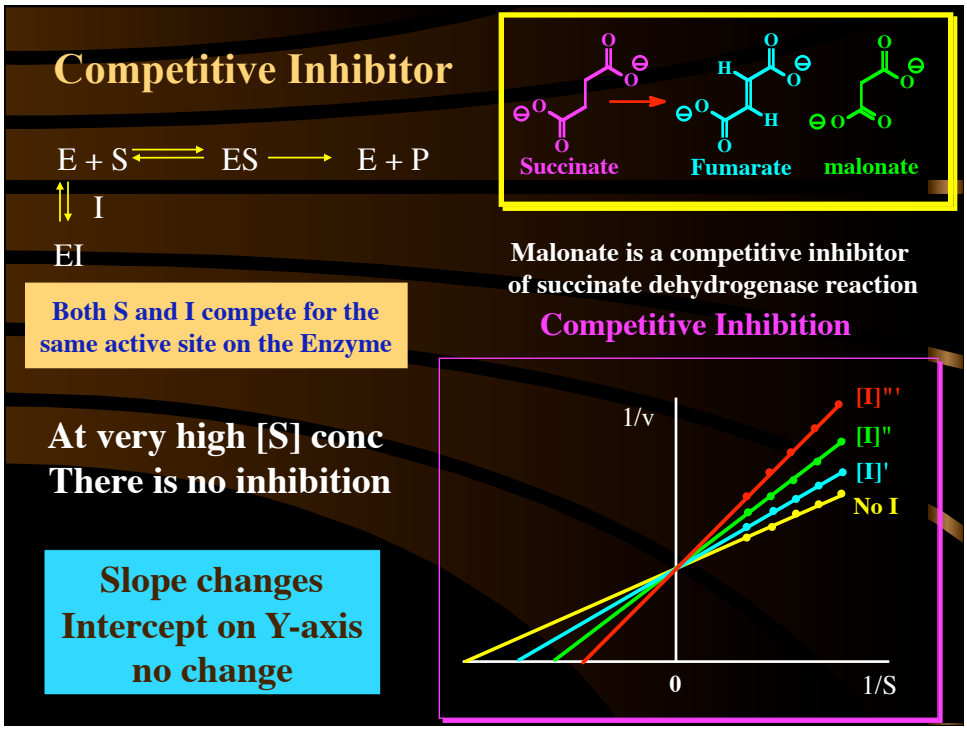
INHIBITORS - DIFFERENT TYPES

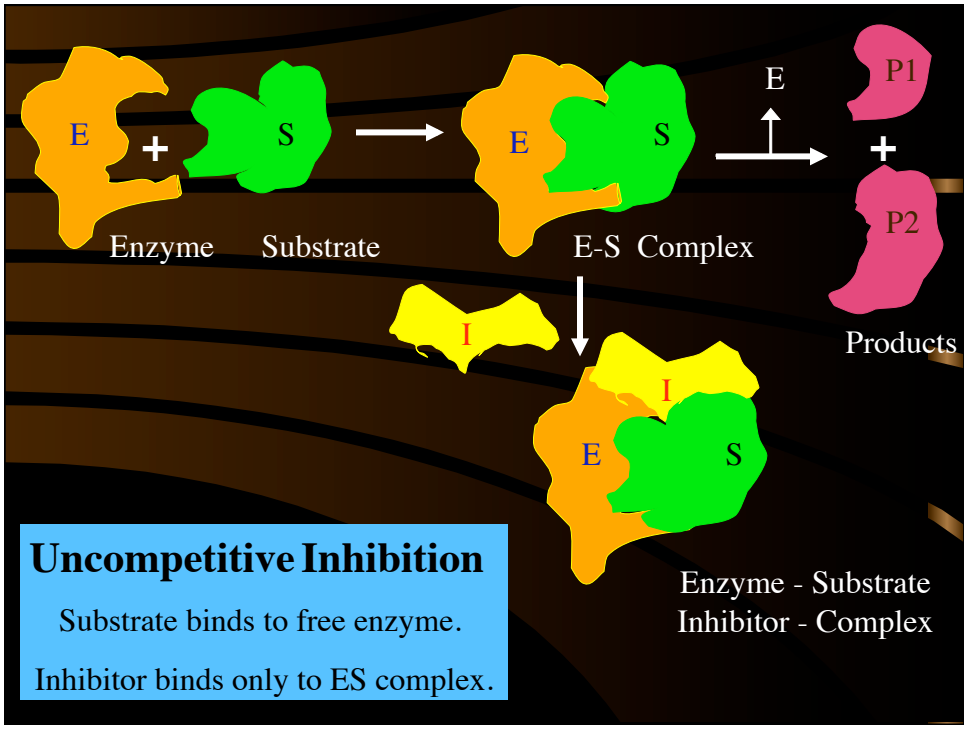


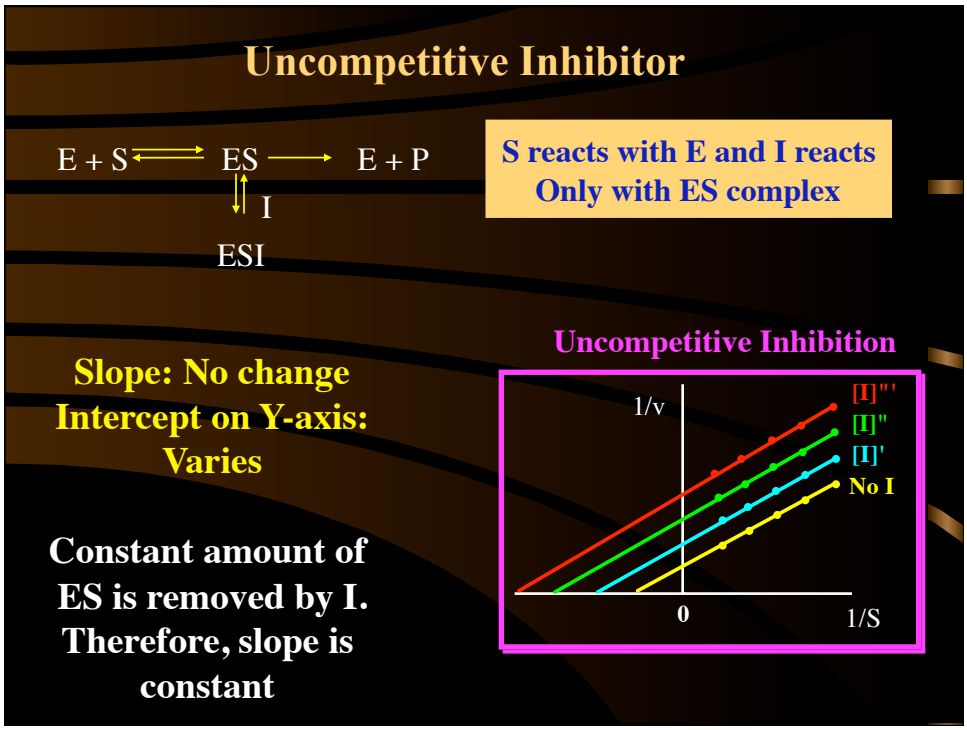

Active site titration of trypsin
p - Nitrophenyl - p' - guanidinobenzoate (NPGB)
FAST
SLOW
Trypsin

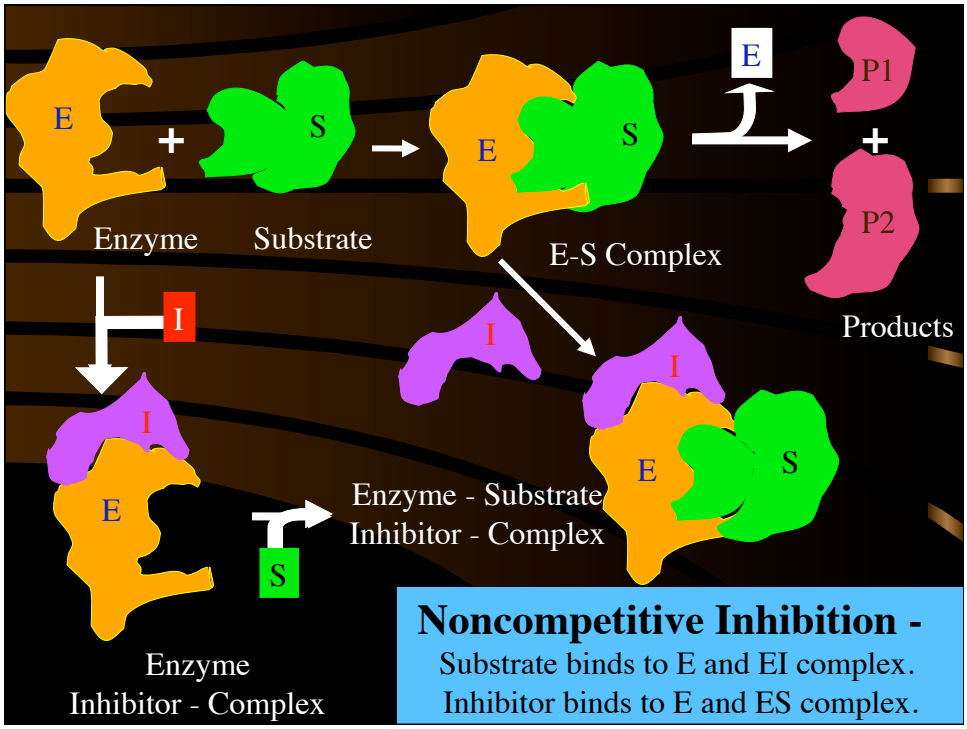

THREE DIFFERENT REVERSIBLE INHIBITORS
REVERSIBLE INHIBITORS
COMPETITIVE
UNCOMPETITIVE
NONCOMPETITIVE

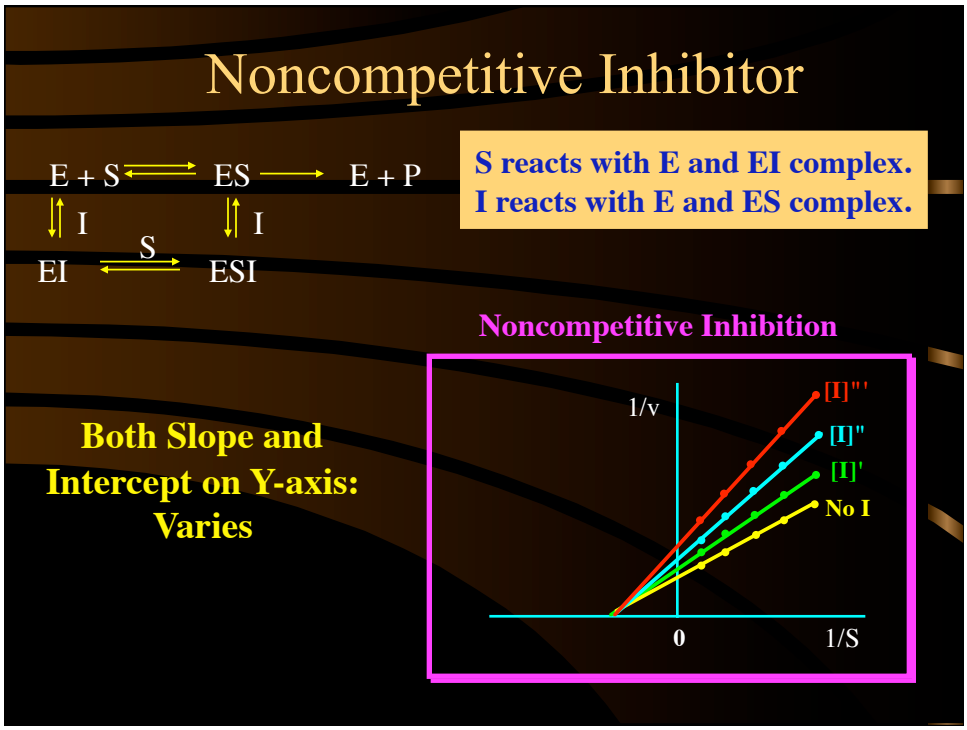

E
S
Substrate
Enzyme
E-S Complex
E
P1
P2
Products
I
E I
Enzyme - inhibitor complex
Succinate
Fumarate
malonate
Competitive Inhibition
Both Substrate and Inhibitor compete for the same active site on the enzyme

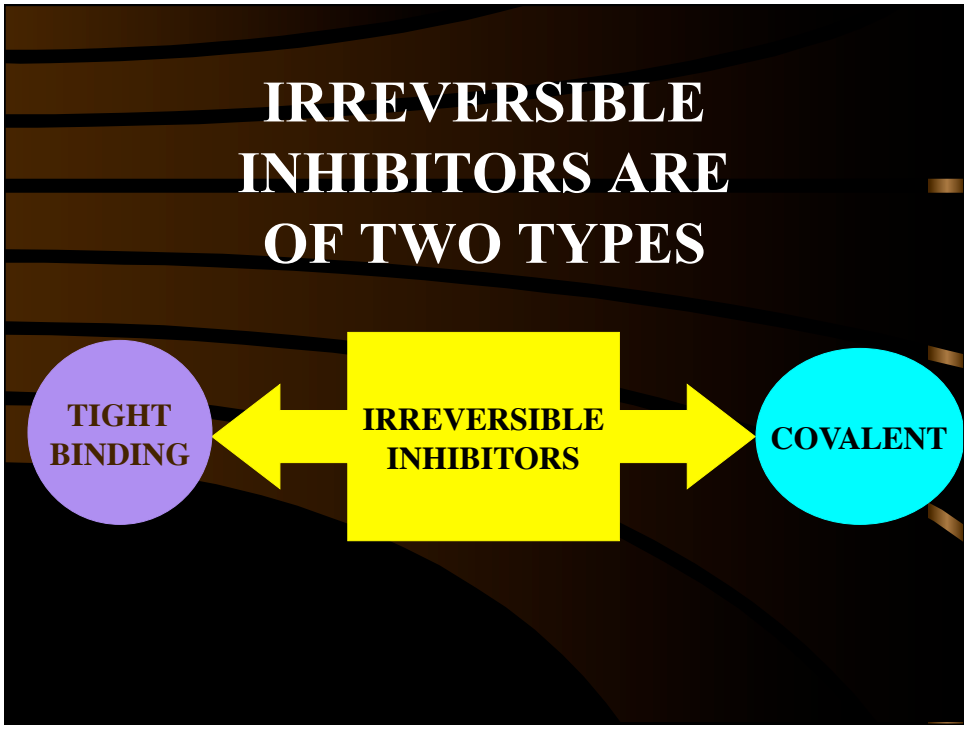

Competitive Inhibitor
E + S ⇌ ES → E + P
I
EI
Both S and I compete for the same active site on the Enzyme
At very high [S] conc
There is no inhibition
Slope changes
Intercept on Y-axis
no change
Succinate
Fumarate
malonate
Malonate is a competitive inhibitor of succinate dehydrogenase reaction
Competitive Inhibition
1/v
[I]"'
[I]"
[I]'
No I
0
1/S

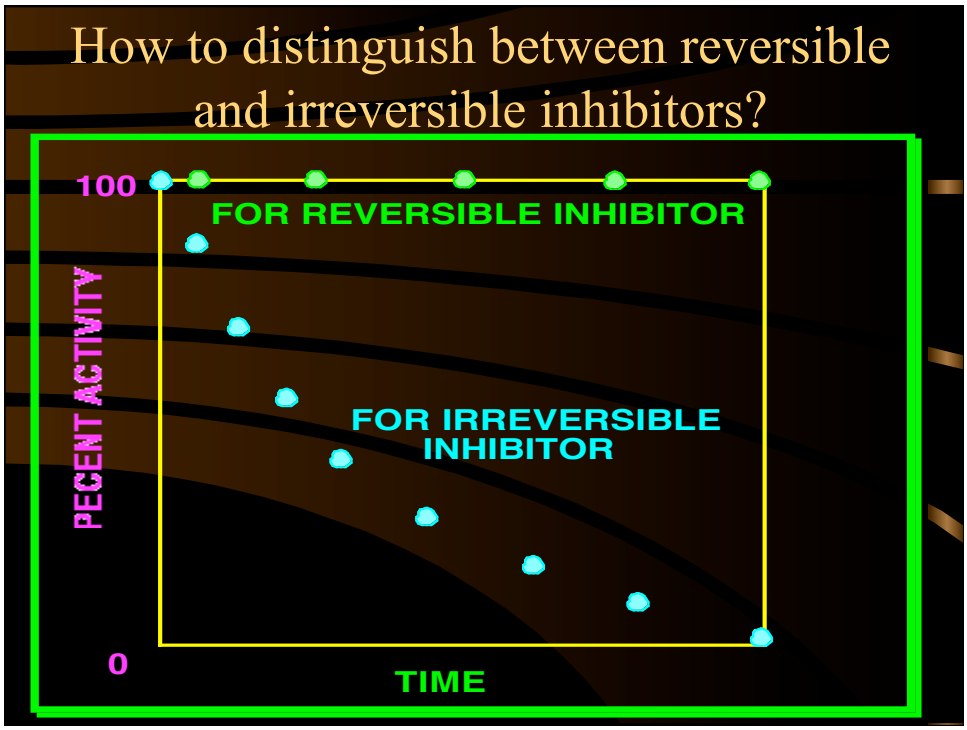

E
S
Enzyme
Substrate
E
S
E-S Complex
E
P1
P2
Products
I
I
E
S
Uncompetitive Inhibition
Substrate binds to free enzyme.
Inhibitor binds only to ES complex.
Enzyme - Substrate
Inhibitor - Complex


Uncompetitive Inhibitor
E + S
ES
E + P
I
ESI
S reacts with E and I reacts
Only with ES complex
Slope: No change
Intercept on Y-axis:
Varies
Constant amount of
ES is removed by I.
Therefore, slope is
constant
Uncompetitive Inhibition
1/v
[I]"'
[I]"
[I]'
No I
0
1/S

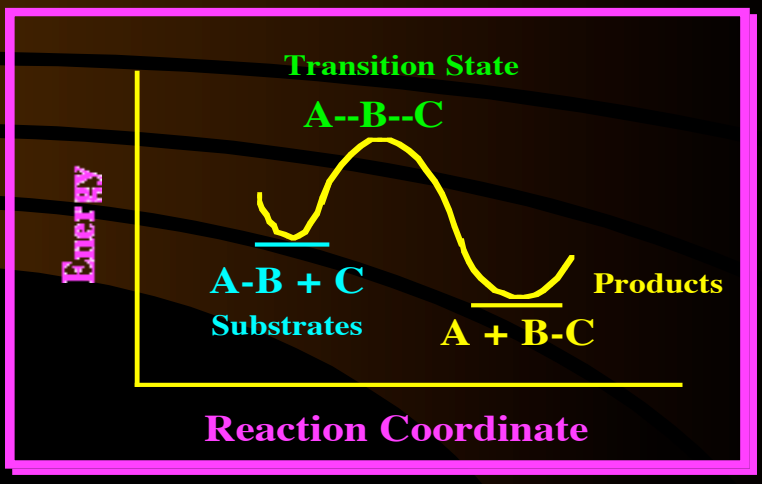

E
S
E
S
E
P1
P2
Enzyme
Substrate
E-S Complex
Products
I
I
I
E
S
E
S
Enzyme - Substrate
Inhibitor - Complex
Enzyme
Inhibitor - Complex
Noncompetitive Inhibition -
Substrate binds to E and EI complex.
Inhibitor binds to E and ES complex.


Noncompetitive Inhibitor
E + S
ES
E + P
I
I
S
EI
ESI
S reacts with E and EI complex.
I reacts with E and ES complex.
Noncompetitive Inhibition
Both Slope and
Intercept on Y-axis:
Varies
1/v
[I]"'
[I]"
[I]'
No I
0
1/S


IRREVERSIBLE INHIBITORS ARE OF TWO TYPES
TIGHT BINDING
IRREVERSIBLE INHIBITORS
COVALENT


How to distinguish between reversible and irreversible inhibitors?
100
FOR REVERSIBLE INHIBITOR
PECENT ACTIVITY
FOR IRREVERSIBLE INHIBITOR
0
TIME

Tight Binding Inhibitors - Transition State Analogs

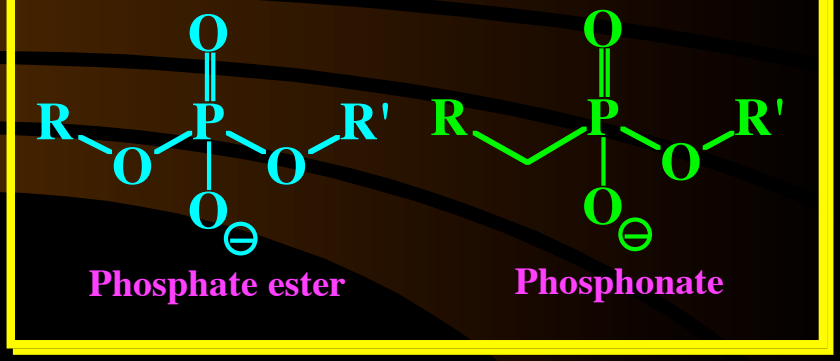


Transition State Analogs are Potent Enzyme Inhibitors

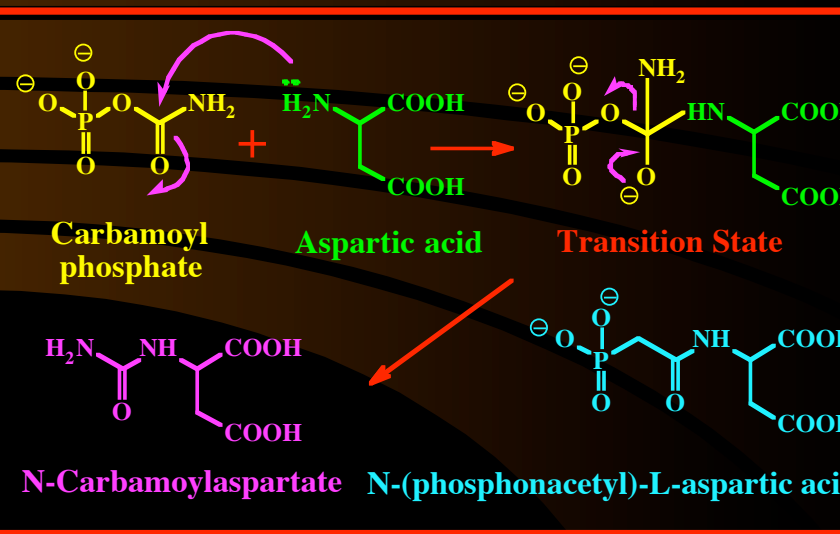
Compounds resembling the transition state of enzyme catalyzed reactions should be effective inhibitors of enzymes.

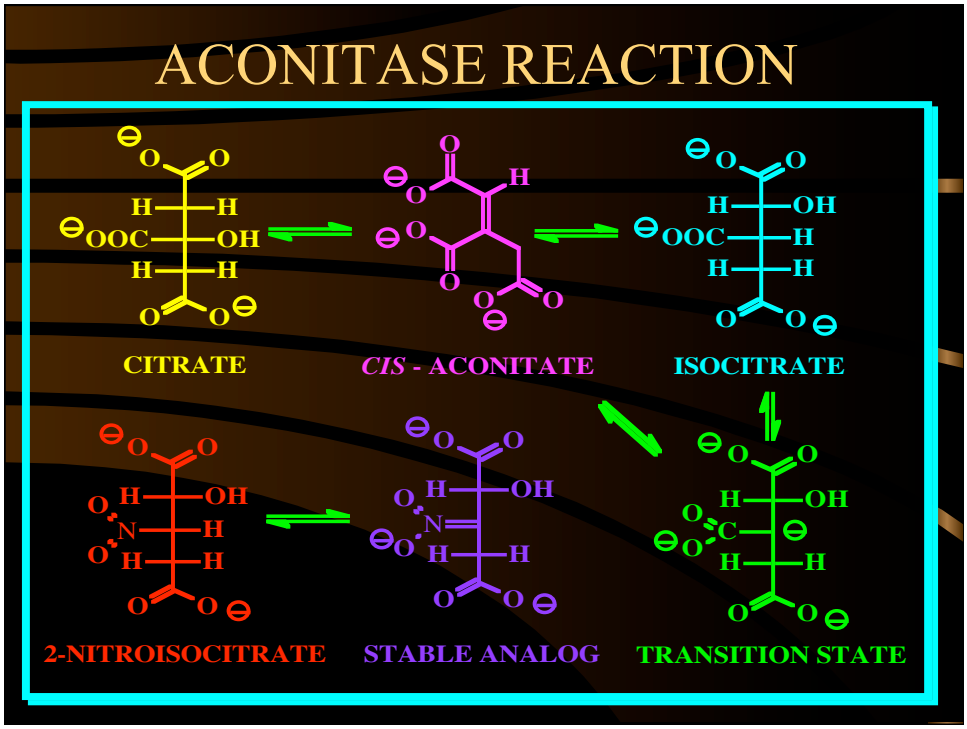
- Linus Pauling (1946)

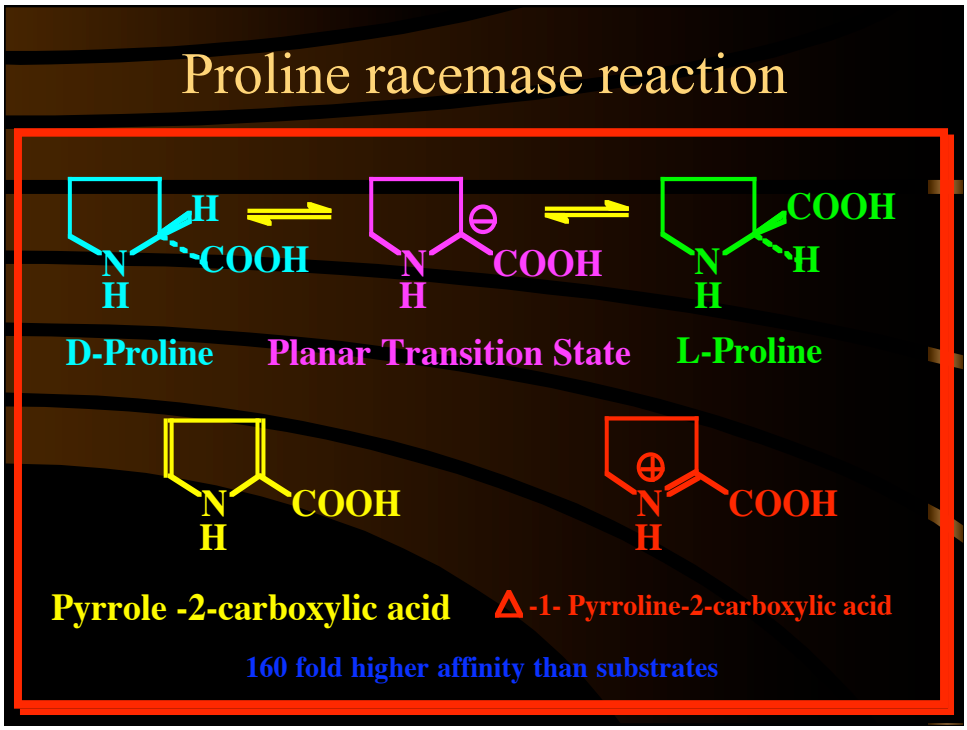
Phosphonates are good analogs of phosphate esters

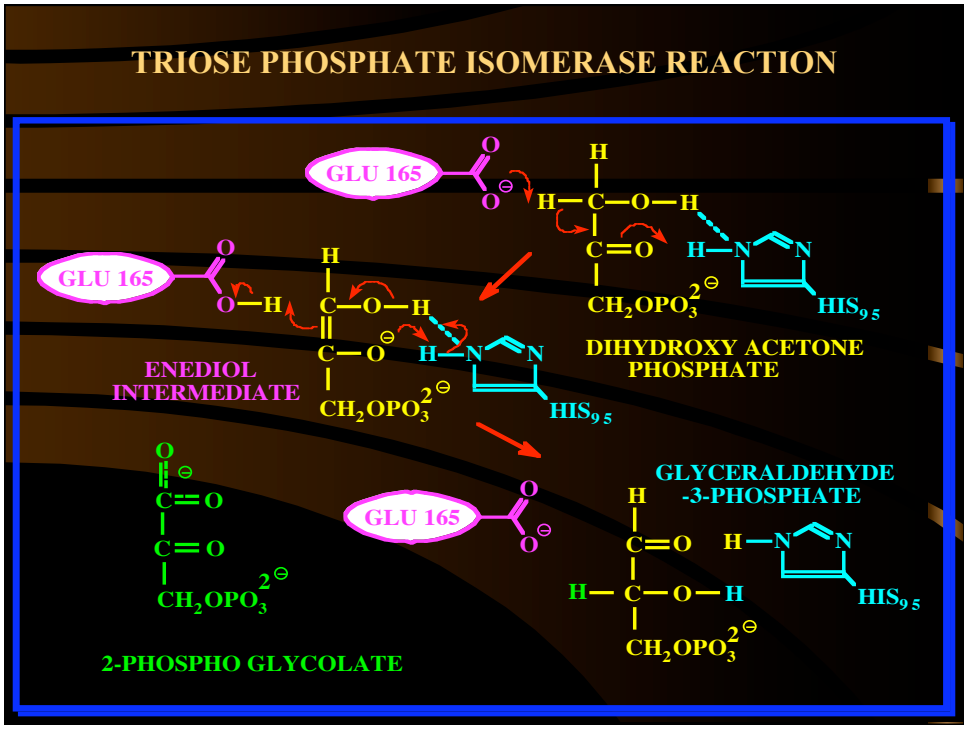


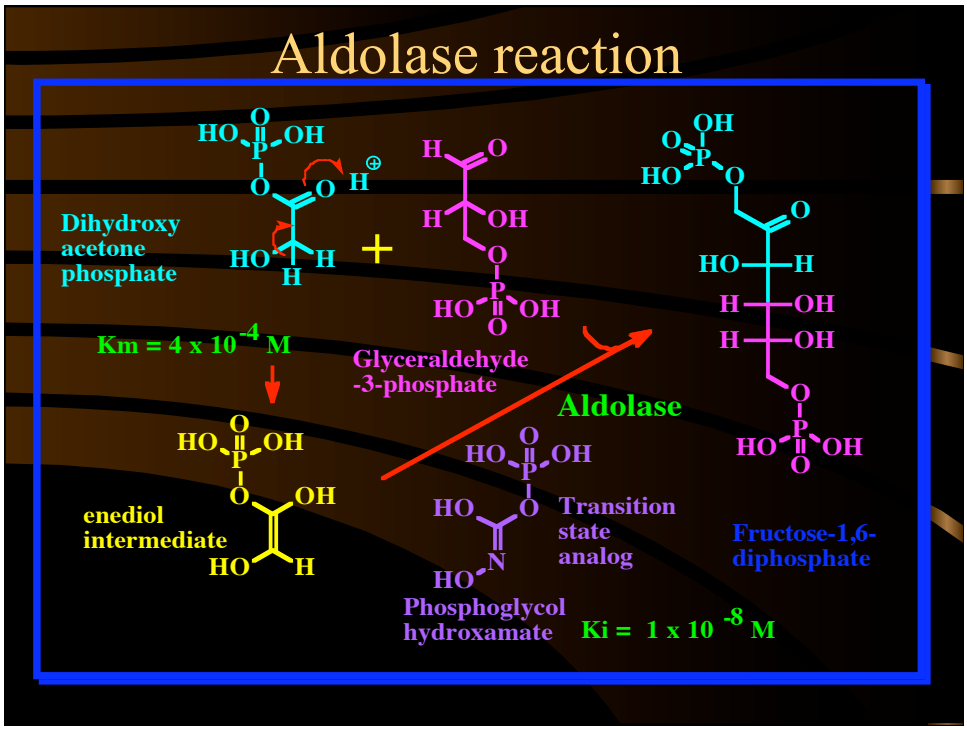
Aspartate transcarbamoylase and its transition state analog

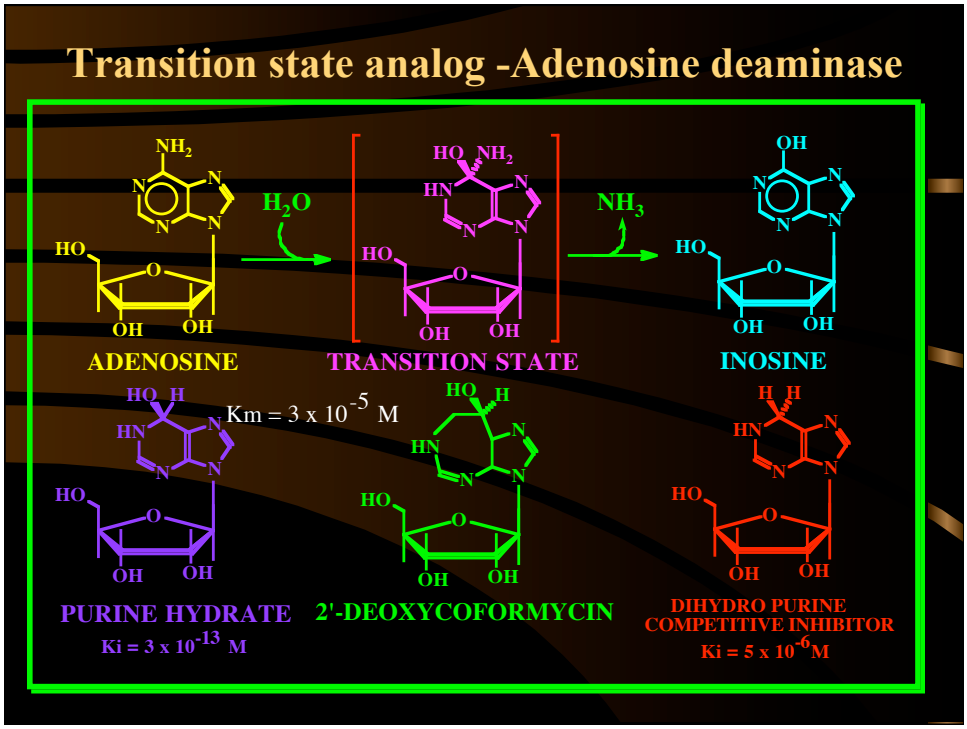


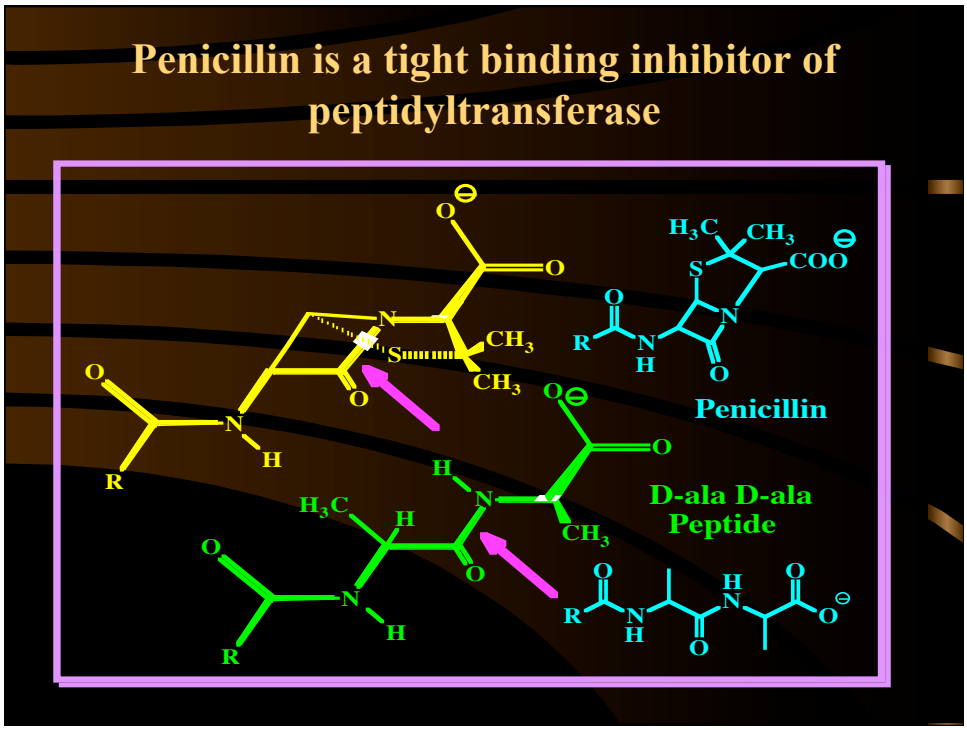

ACONITASE REACTION
CITRATE
CIS - ACONITATE
ISOCITRATE
2-NITROISOCITRATE
STABLE ANALOG
TRANSITION STATE


Proline racemase reaction
D-Proline
Planar Transition State
L-Proline
Pyrrole -2-carboxylic acid
Δ -1- Pyrroline-2-carboxylic acid
160 fold higher affinity than substrates

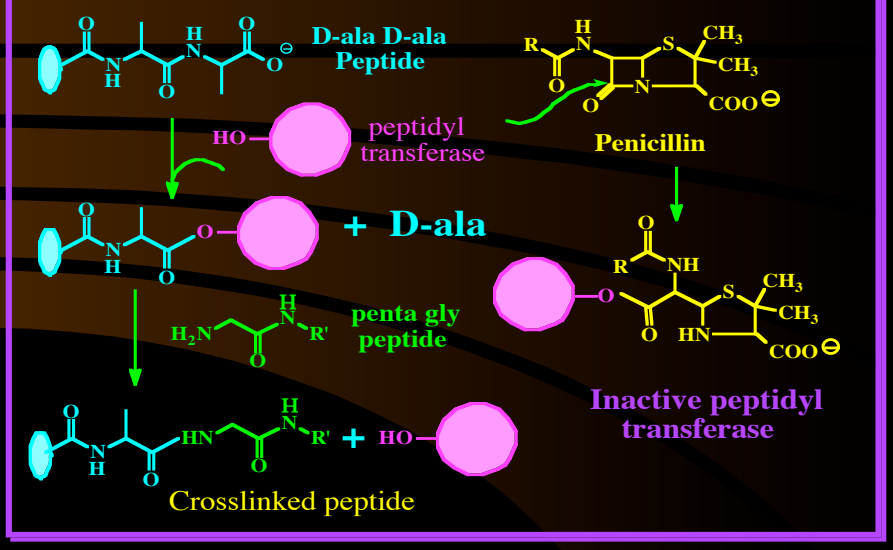

TRIOSE PHOSPHATE ISOMERASE REACTION
GLU 165
ENEDIOL INTERMEDIATE
DIHYDROXY ACETONE PHOSPHATE
GLYCERALDEHYDE -3-PHOSPHATE
2-PHOSPHO GLYCOLATE
HIS95


Aldolase reaction
Dihydroxy acetone phosphate
Km = 4 x 10-4 M
Glyceraldehyde -3-phosphate
Aldolase
enediol intermediate
Transition state analog
Fructose-1,6-diphosphate
Phosphoglycol hydroxamate
Ki = 1 x 10-8 M


Transition state analog -Adenosine deaminase
ADENOSINE
TRANSITION STATE
INOSINE
Km = 3 x 10-5 M
PURINE HYDRATE
Ki = 3 x 10-13 M
2'-DEOXYCOFORMYCIN
DIHYDRO PURINE
COMPETITIVE INHIBITOR
Ki = 5 x 10-6M

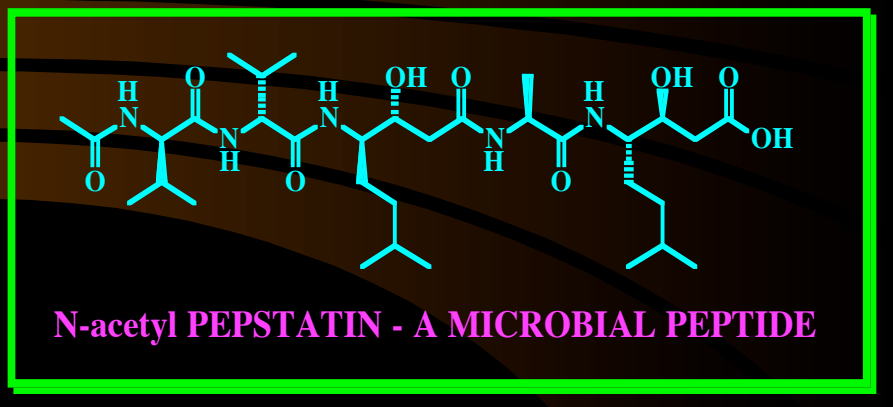

Penicillin is a tight binding inhibitor of peptidyltransferase
Penicillin
D-ala D-ala
Peptide

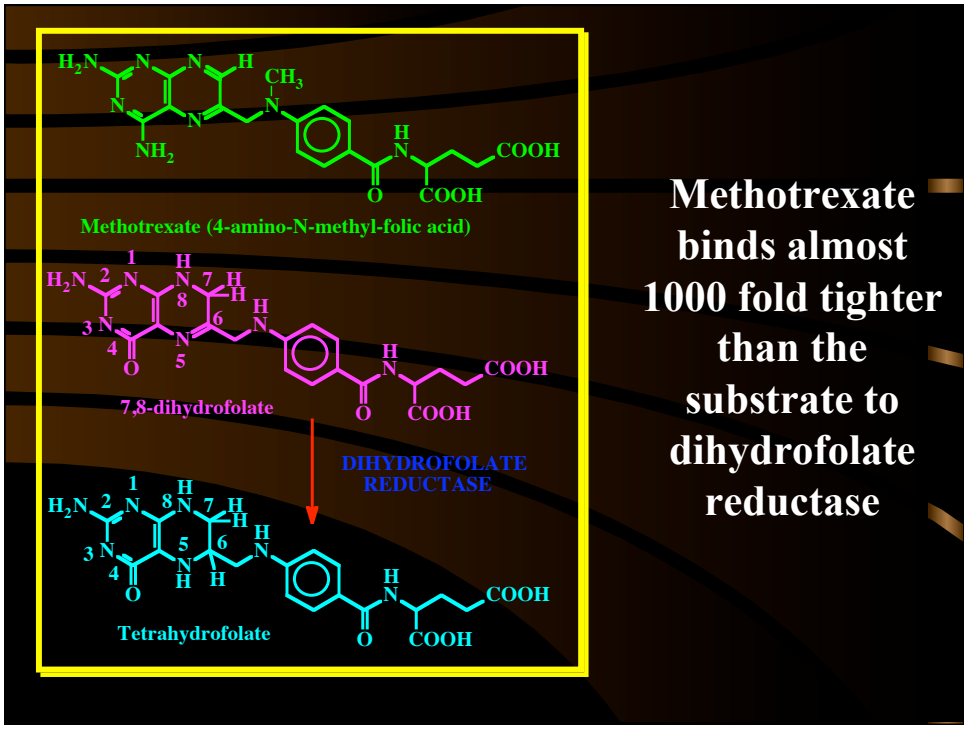
Penicillin inhibits transpeptidase reaction

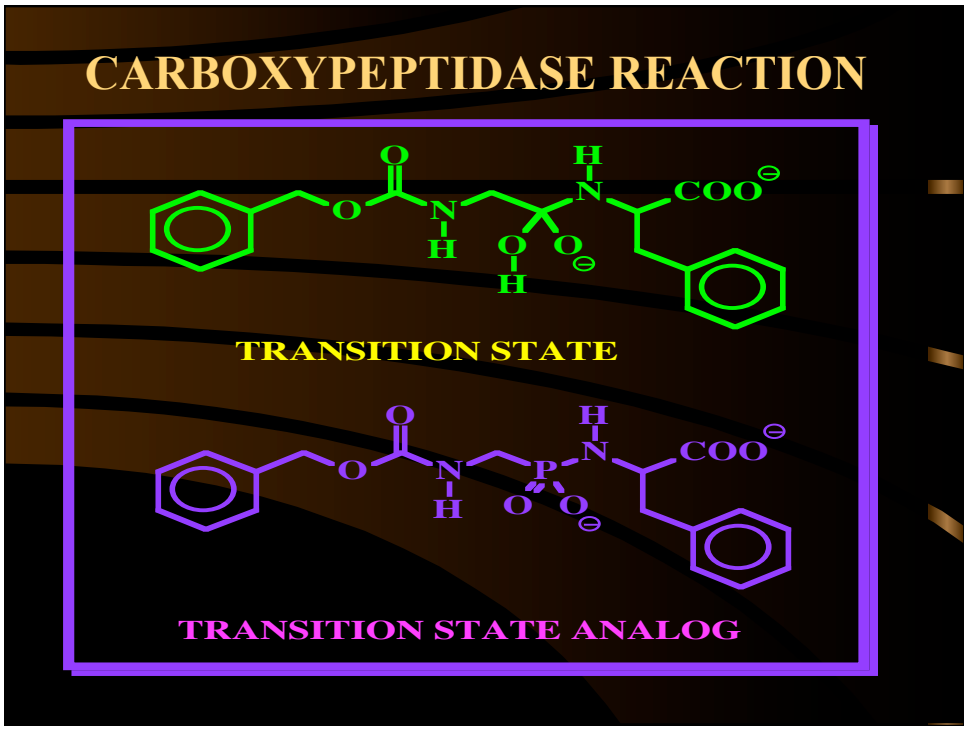


Pepstatin is a potent transition state analog of aspartyl proteases

(Hydroxyethylene group inside the peptide)



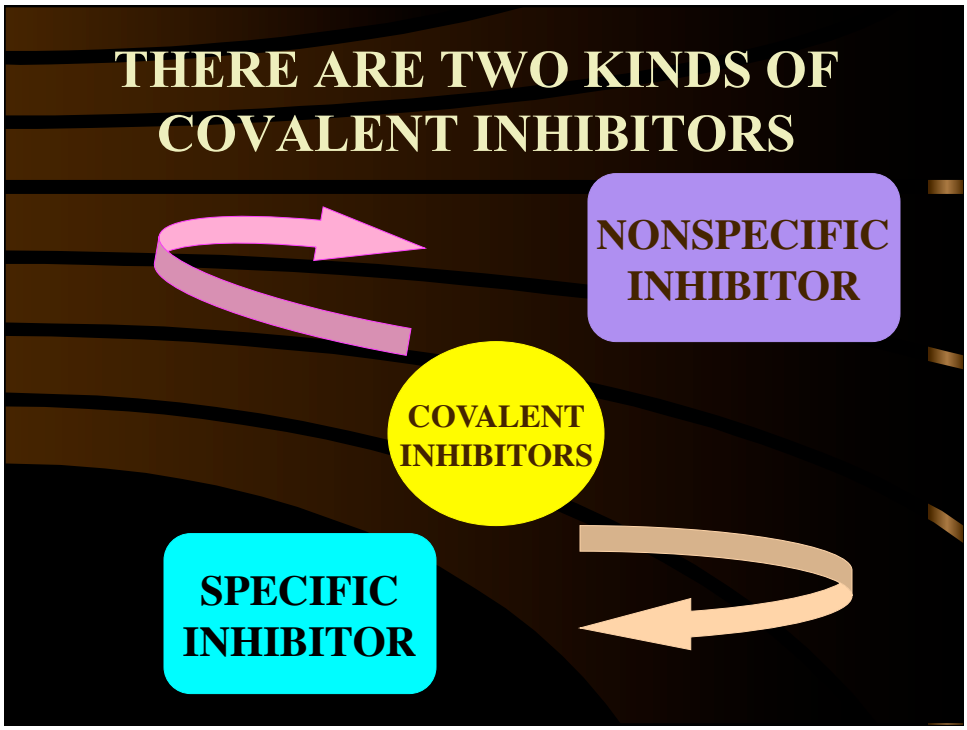

Methotrexate (4-amino-N-methyl-folic acid)
7,8-dihydrofolate
DIHYDROFOLATE REDUCTASE
Tetrahydrofolate
Methotrexate binds almost 1000 fold tighter than the substrate to dihydrofolate reductase


CARBOXYPEPTIDASE REACTION
TRANSITION STATE
TRANSITION STATE ANALOG

OTHER TRANSITION STATE INHIBITORS

- **LYSOZYME - LACTONE**
- **RIBONUCLEASE - URIDINE VANADATE**
- **ISOCITRATE LYASE - 3-NITRO PROPIONATE**
- **RIBULOSE BISPHOSPHATE CARBOXYLASE - CARBOXY ARABINITOL 1,5-BISPHOSPHATE**
- **GLUTAMINE SYNTHETASE - METHIONINE SULFOXIMINE**
- **GAMMA -GLUTAMYL CYSTEINYL SYNTHETASE - S-(n-BUTYL)-HOMOCYSTEINE SULFOXIMINE**

THERE ARE TWO KINDS OF COVALENT INHIBITORS



Nonspecific Covalent Inhibitors

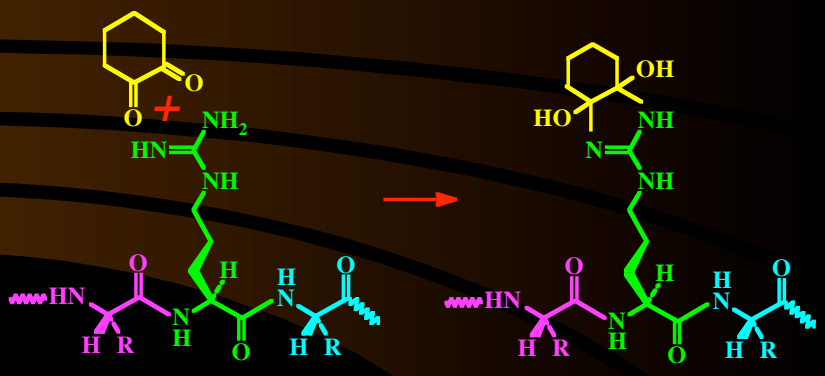
They react invariably with any enzyme as long as
a particular reactive amino acid is present at or near the active site.
Generally the reaction leads to inactivation of the enzyme.

Best examples are active site reagents.

Serine - organophosphorus compounds
Histidine, cysteine and Lysine - Haloacetates, Halomethylketones
Histidine -diethylpyrocarbonate
Arginine - 1,2-diketocyclohexane
Cysteine - Thiol reagents, pCMB, DTNB
Metal ion containing enzymes - cyanide, azide, carbon monoxide
Carbonyl groups - reduction, hydroxylamine
Lysine - anhydrides
Carboxyl groups - esterification.

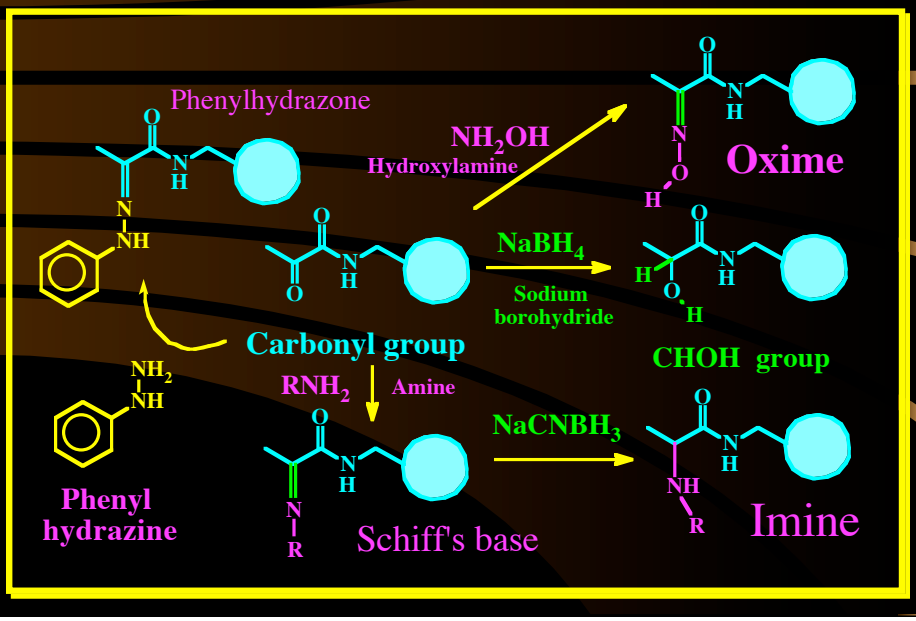
Modification of arginine

Reversible blocking of Arginine by cyclohexadione.
E. L. Smith. *Methods in Enzymology* 47, 156-161 (1977).

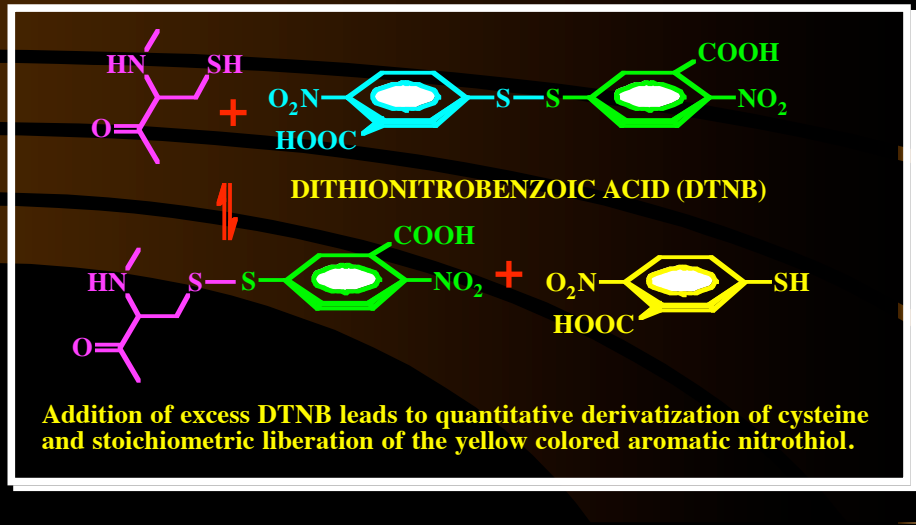


Arginine is modified by 1,2-cyclohexadione (or phenylglyoxal).
The vicinyl dihydroxy adduct can be stabilized by borate.

Carbonyl group modifications



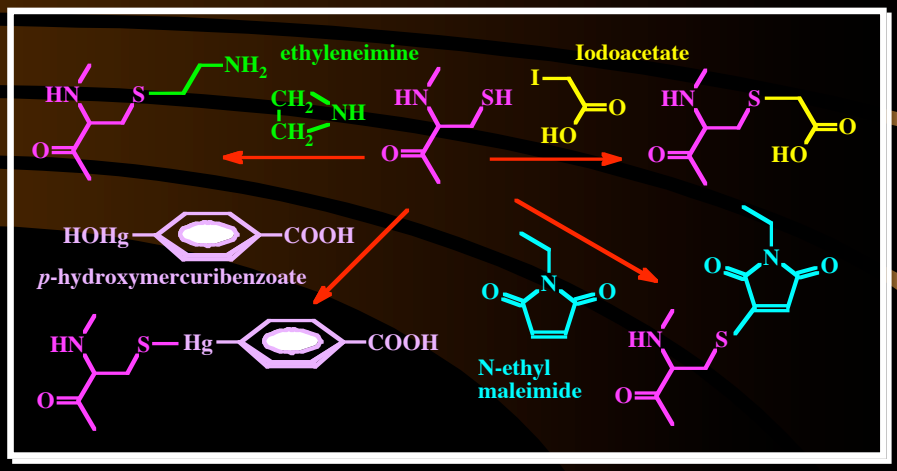
Cysteine modification (DTNB)



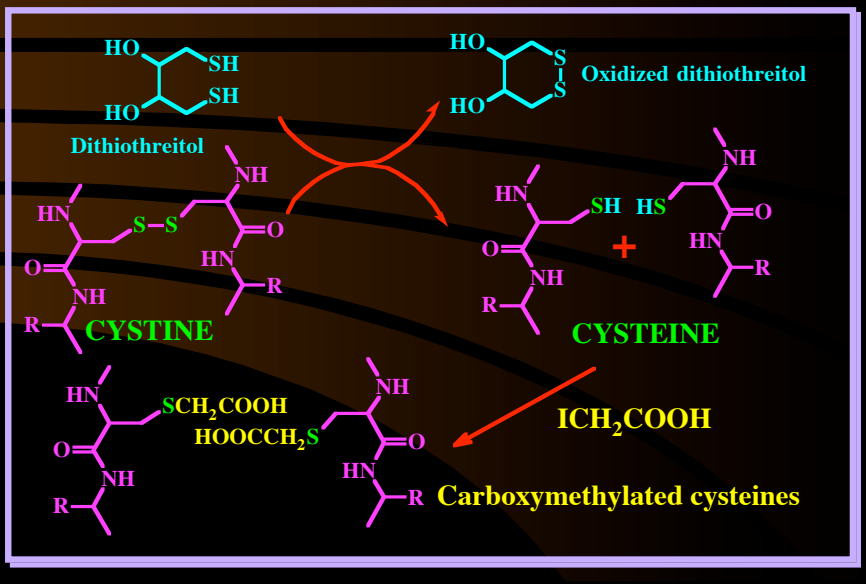
Addition of excess DTNB leads to quantitative derivatization of cysteine and stoichiometric liberation of the yellow colored aromatic nitrothiol.

A number of reagents react with cysteine. Ethyleneimine, NEM, iodoacetate (as well as iodoacetamide) and *p*-hydroxymercuribenzoate

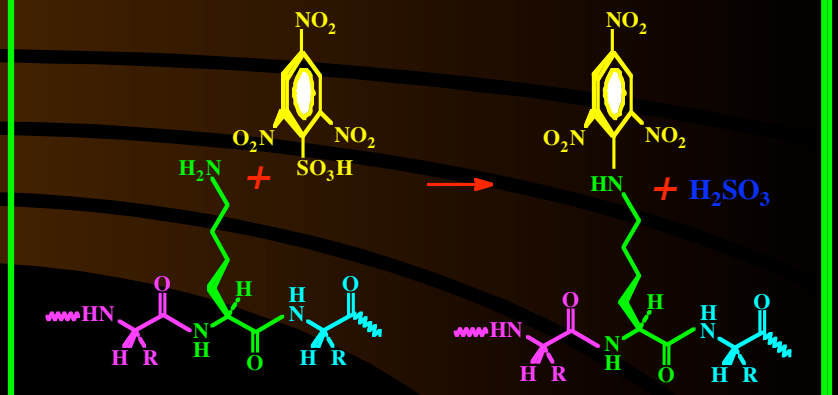
will modify cysteine as shown below .



Dithiothreitol reduces disulfides and iodoacetate blocks them

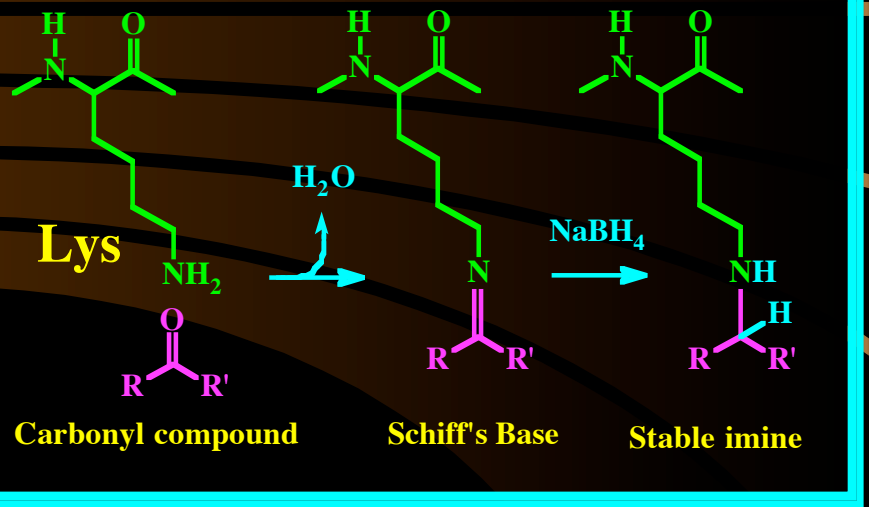


Blocking of lysine with TNBS

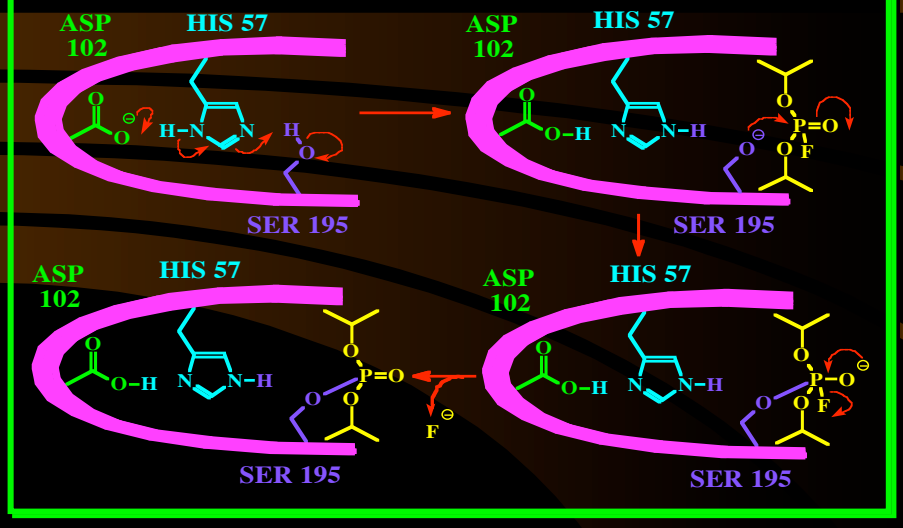


TNBS (2,4,6-trinitrobenzenesulfonic acid arylates lysines.
The product can be quantified at 367 nm.

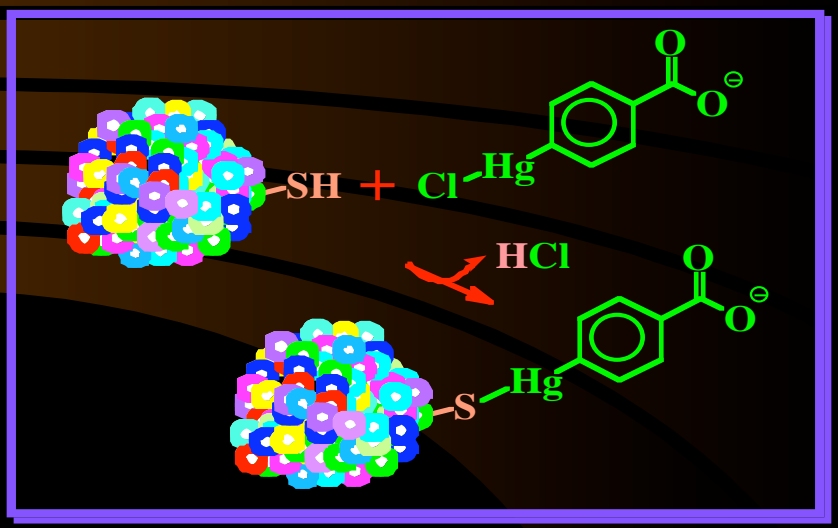
Modification of lysine

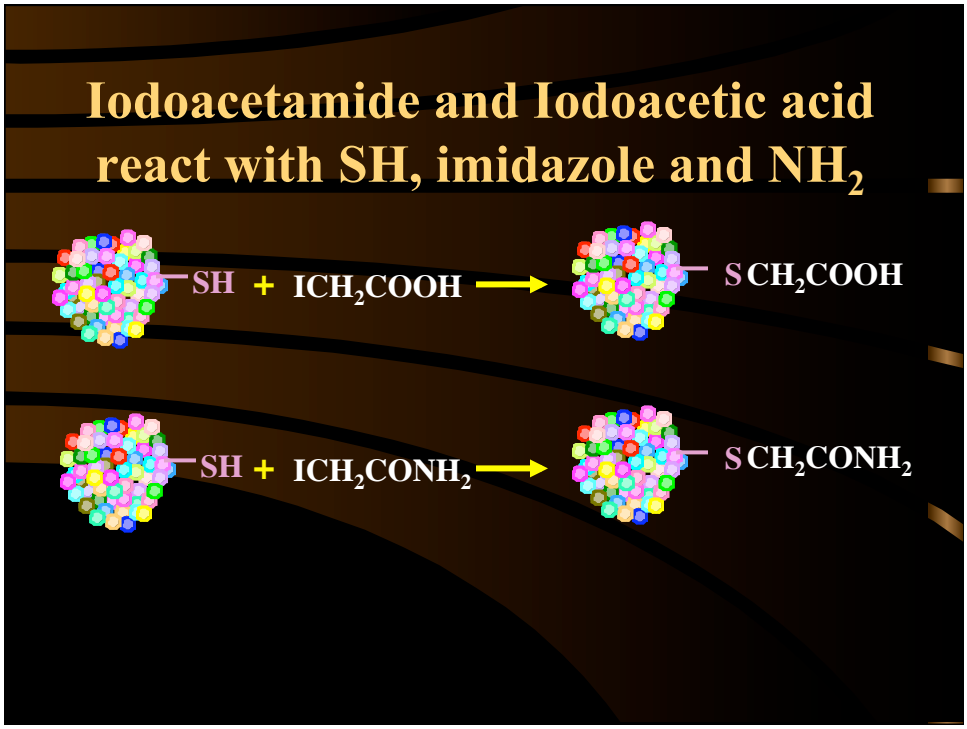


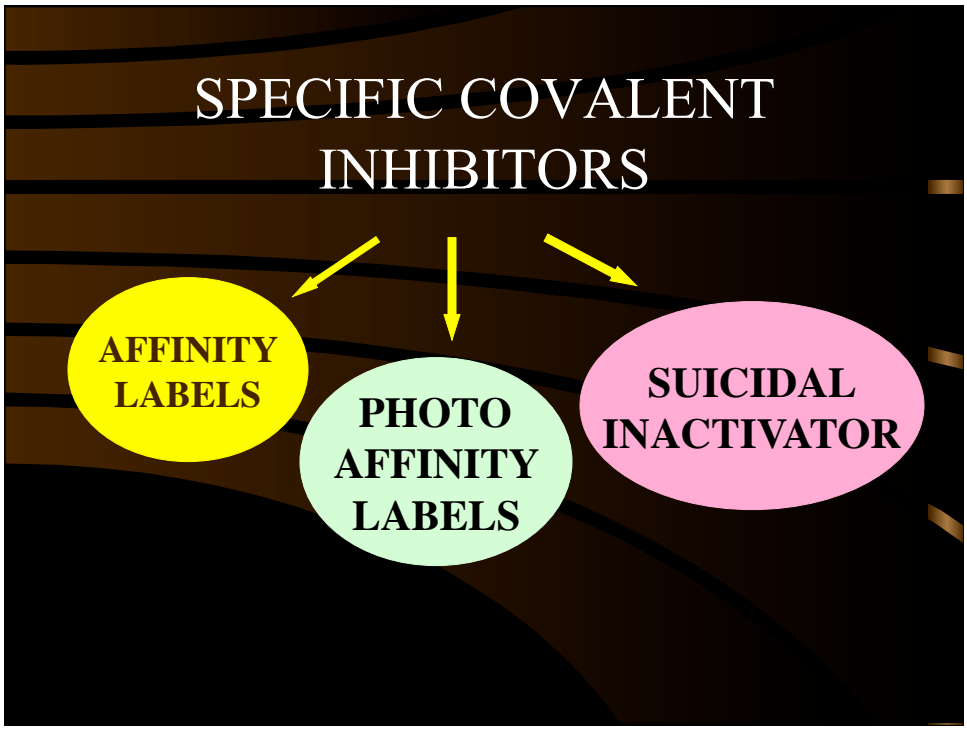
Chymotrypsin inactivation by diisopropylfluorophosphate

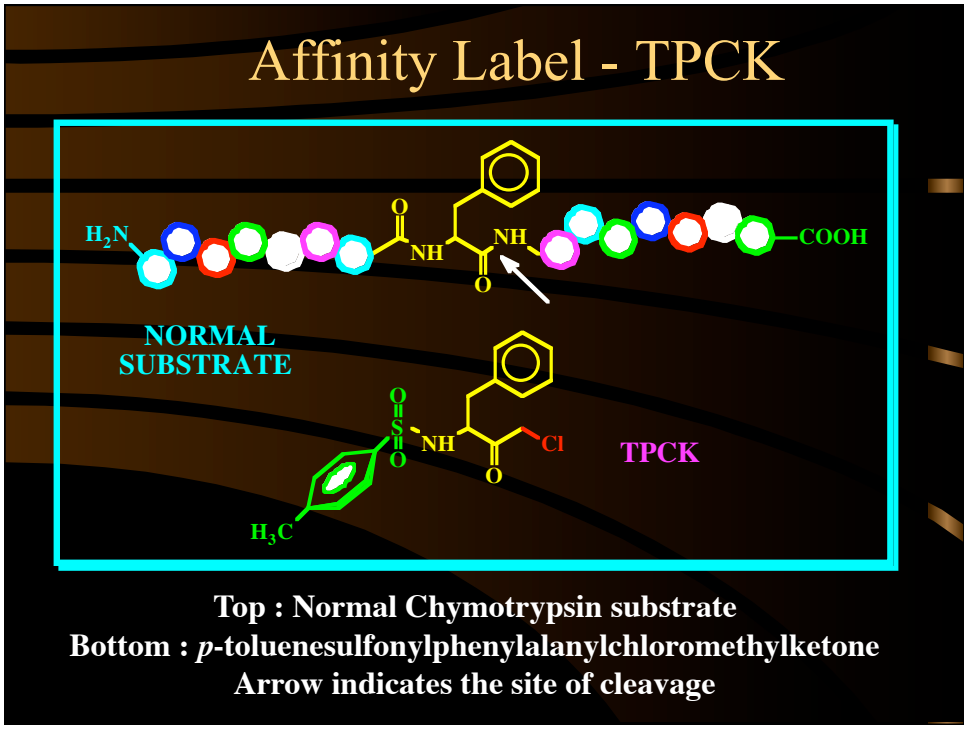


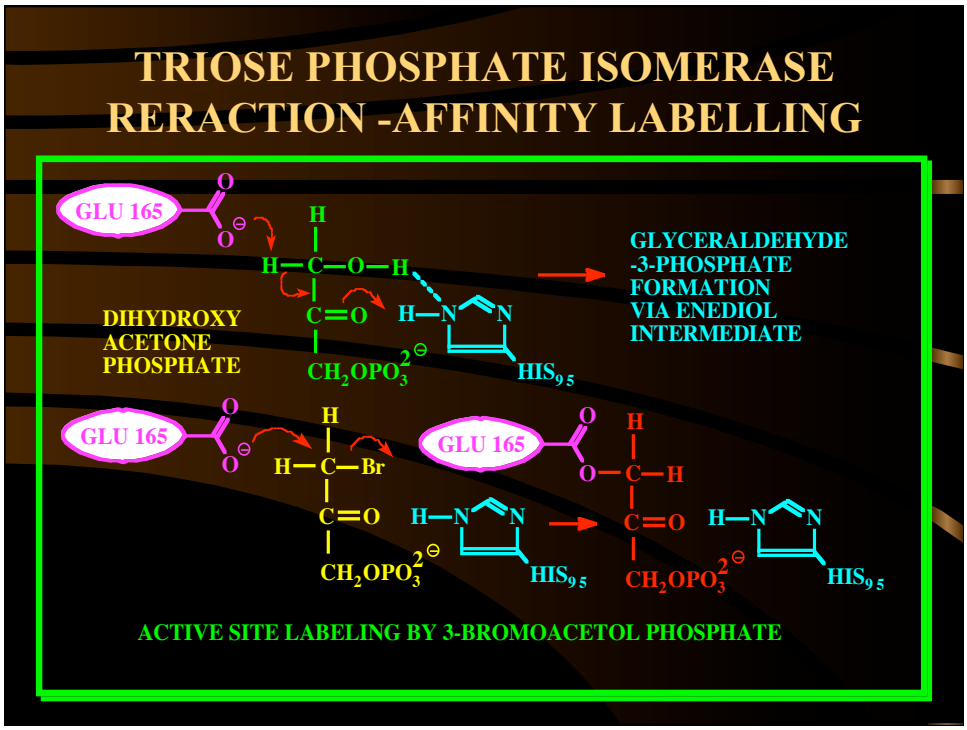
Inactivation of glyceraldehyde-3-phosphate dehydrogenase by *p*-chloromercuribenzoate

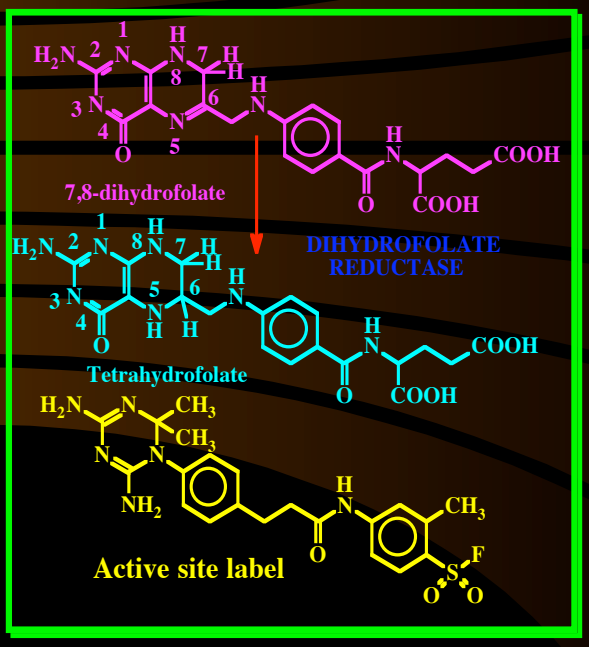



Iodoacetamide and Iodoacetic acid react with SH, imidazole and NH_2
SH + ICH_2COOH
SCH_2COOH
SH + ICH_2CONH_2
SCH_2CONH_2


SPECIFIC COVALENT INHIBITORS
AFFINITY LABELS
PHOTO AFFINITY LABELS
SUICIDAL INACTIVATOR


Affinity Label - TPCK
NORMAL SUBSTRATE
TPCK
Top : Normal Chymotrypsin substrate
Bottom : p-toluenesulfonylphenylalanylchloromethylketone
Arrow indicates the site of cleavage


TRIOSE PHOSPHATE ISOMERASE
RERACTION -AFFINITY LABELLING
GLU 165
DIHYDROXY ACETONE PHOSPHATE
GLYCERALDEHYDE -3-PHOSPHATE FORMATION VIA ENEDIOL INTERMEDIATE
ACTIVE SITE LABELING BY 3-BROMOACETOL PHOSPHATE



Affinity label for dihydro-folate reductase

PHOTOAFFINITY LABEL

Attach a photoreactive group such as diazoacetyl group to a substrate analog.

Allow the enzyme bind to the photoaffinity label.

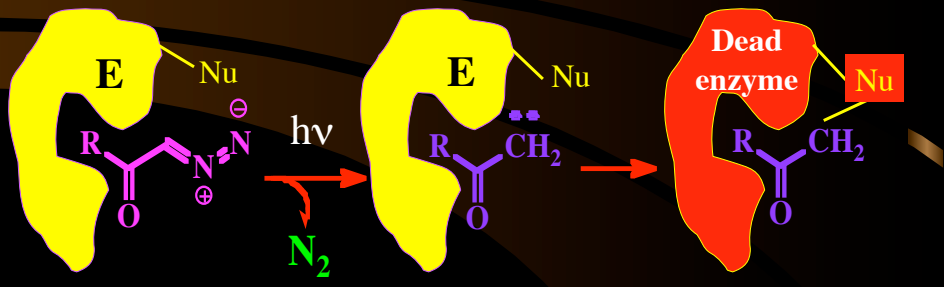
Shine light on the complex.

The photolabile group is activated and a reactive intermediate is generated.

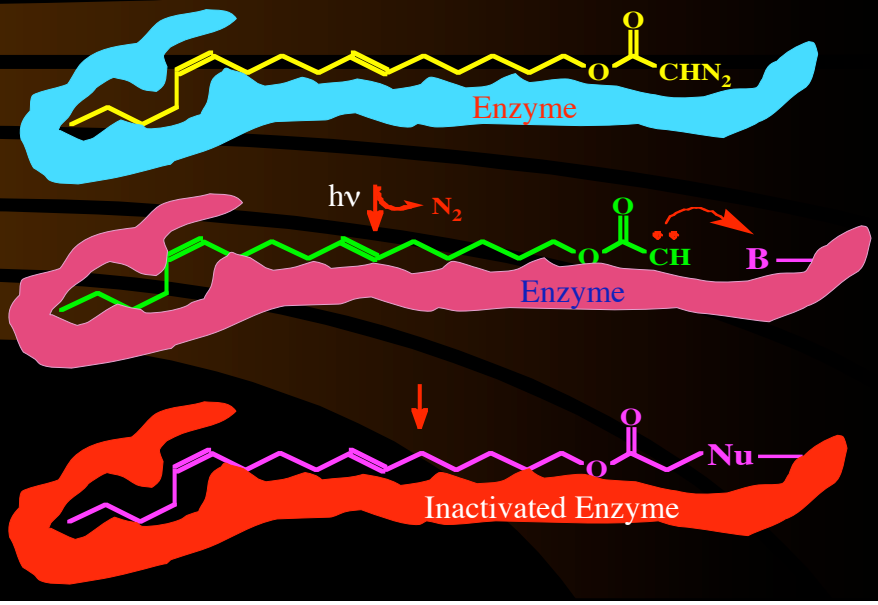
The reactive intermediate rapidly inactivates the enzyme by binding to essential amino acids near near the active site.

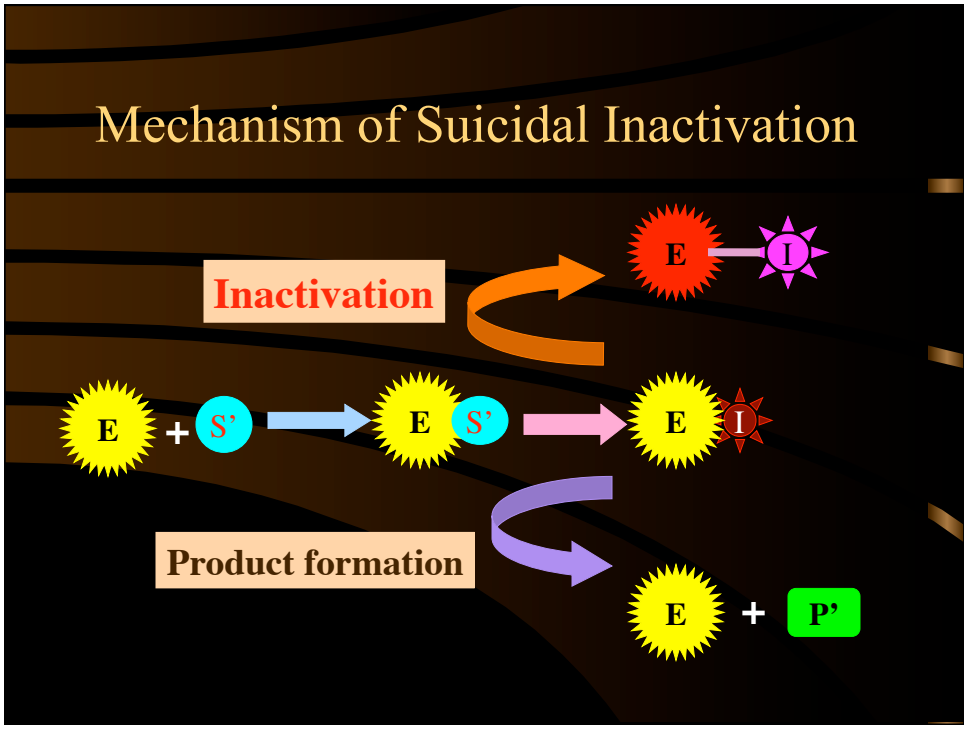
PHOTOAFFINITY LABEL - HOW IT WORKS

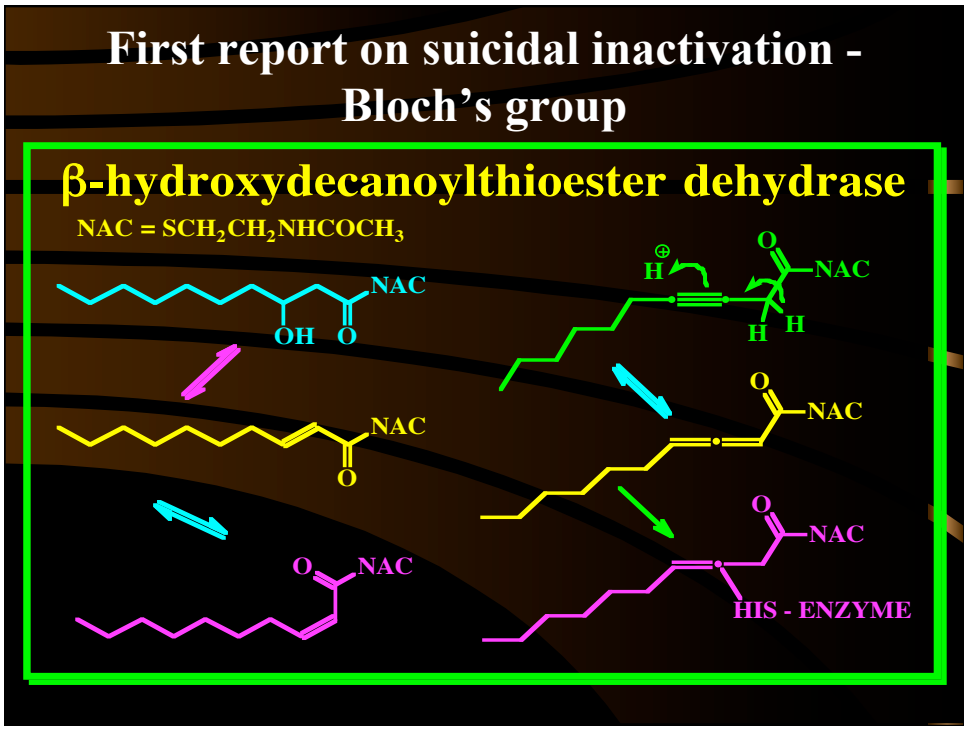
Step 1. Photoaffinity label binds to the protein.
at a site directed by the substituent group (R).
Step 2. Light is shined on the complex to eliminate the
photo labile group.
Step 3. The reactive group generated attaches itself
to the nearly nucleophile killing the protein.



6E, 11Z-hexadecadienyl diazoacetate interaction with pheromone binding protein




Mechanism of Suicidal Inactivation
Inactivation
E
I
E
+
S'
E
S'
E
I
Product formation
E
+
P'


First report on suicidal inactivation -
Bloch's group
β-hydroxydecanoylthioester dehydrase
NAC = SCH2CH2NHCOCH3
NAC
OH
O
NAC
O
O
NAC
H
H
H
O
NAC
O
NAC
HIS - ENZYME

Criteria for suicidal inactivation

- **Inhibitor should be unreactive.**
- **It should become reactive only after activation by enzymatic action.**
- **Conversion of unreactive to reactive inhibitor should be due to specific catalytic function of the enzyme.**
- **Nonspecific interaction should be minimum.**

(a) Nontarget enzyme reaction

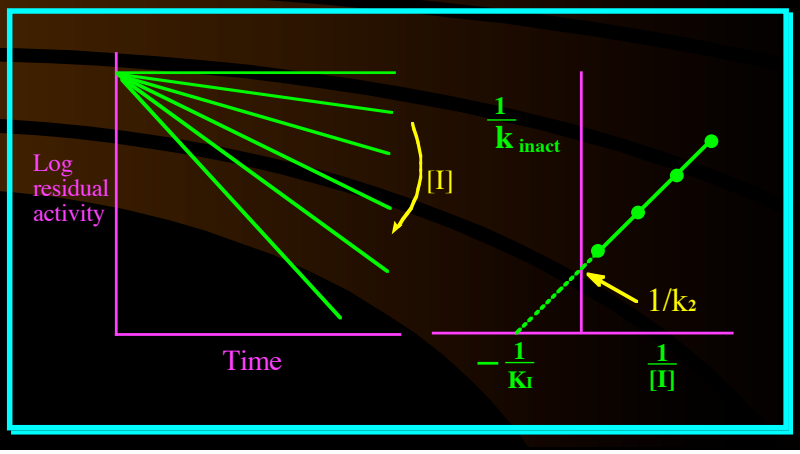
(b) Escape of reactive intermediate before reaction.

How to identify suicidal inactivators?

- Best way to identify is the characterization of enzyme inhibitor complex by physico-chemical studies.
- But this could be difficult in some cases. So one needs some easy techniques to identify the suicidal inactivators.

Kinetic experiments can identify suicidal inactivators

- **The loss of enzyme activity should follow time dependent first order kinetics at fixed concentration of inhibitor.**
- **(If a reactive inhibitor comes out and then reacts with the enzyme, it will obviously follow second order kinetics. More over second addition of enzyme to this reaction mixture will cause an increased rate of inactivation due to accumulated inhibitor. Also addition of nucleophiles will reduce the rate of inactivation in this mode).**



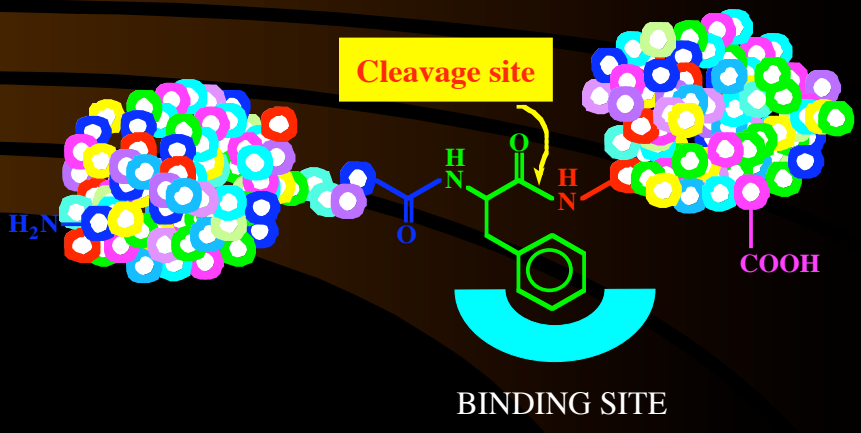
Kinetics of inactivation

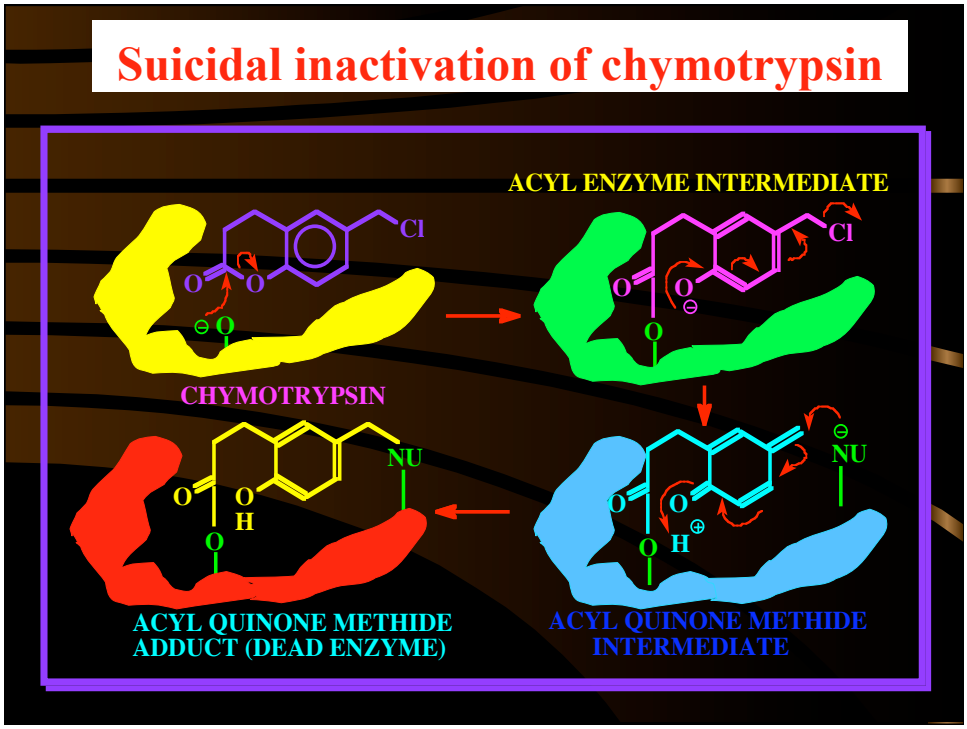
- The rate of inactivation should follow Michaelis Menten type kinetics.
- Substrate (or competitive inhibitor) should protect the enzyme from inactivation.

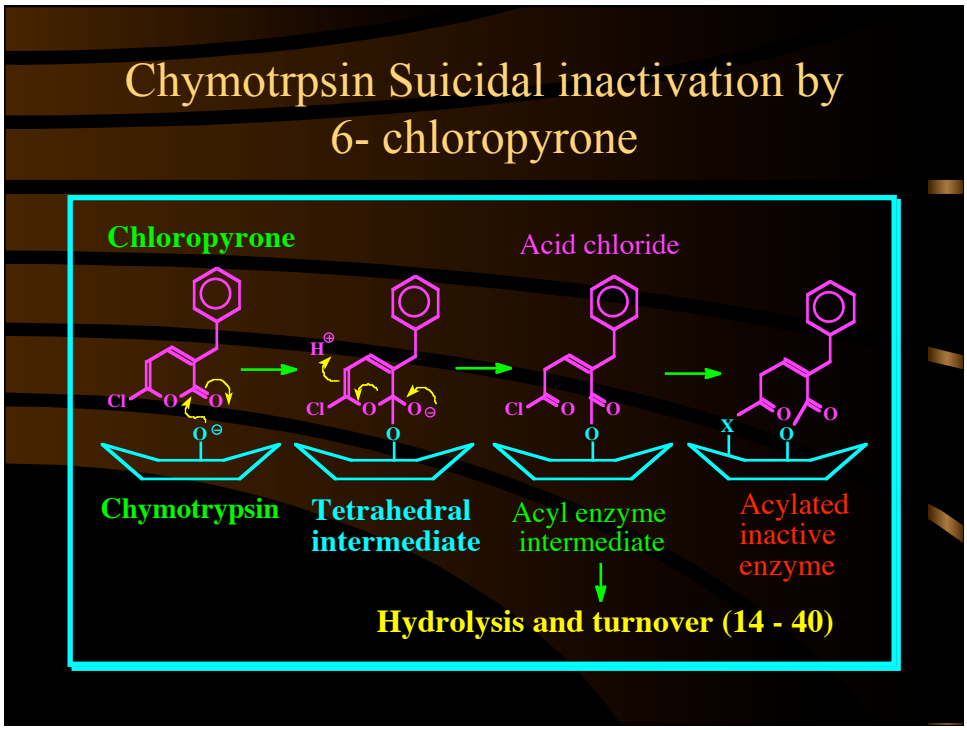
Inactivation Kinetics

- Inactivation Should be irreversible. (Show by dialysis, gel filtration etc.,)
- Inactivator should be bound covalently to the enzyme (show with radiolabel or other techniques).
- Stoichiometry of binding should be one to one mole.
- Partitioning between catalysis and inactivation should be determined.

Chymotrypsin is an endopeptidase that cleaves proteins on the carboxyl side of aromatic amino acids (Phe, Tyr, Trp) and Leu.

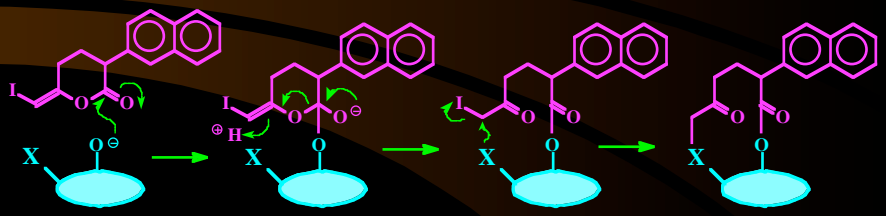



Suicidal inactivation of chymotrypsin
ACYL ENZYME INTERMEDIATE
CHYMOTRYPSIN
ACYL QUINONE METHIDE ADDUCT (DEAD ENZYME)
ACYL QUINONE METHIDE INTERMEDIATE


Chymotrpsin Suicidal inactivation by 6- chloropyrone
Chloropyrone
Acid chloride
Chymotrypsin
Tetrahedral intermediate
Acyl enzyme intermediate
Acylated inactive enzyme
Hydrolysis and turnover (14 - 40)

Highly specific chymotrypsin inactivator

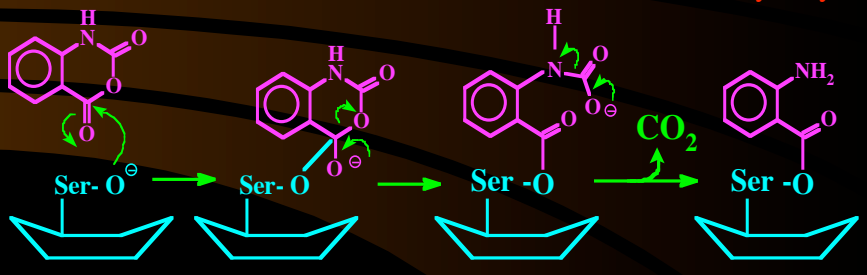
6-iodomethylene napphthyl tetrahydropyran-2-one (1.7 turnover per inactivation)

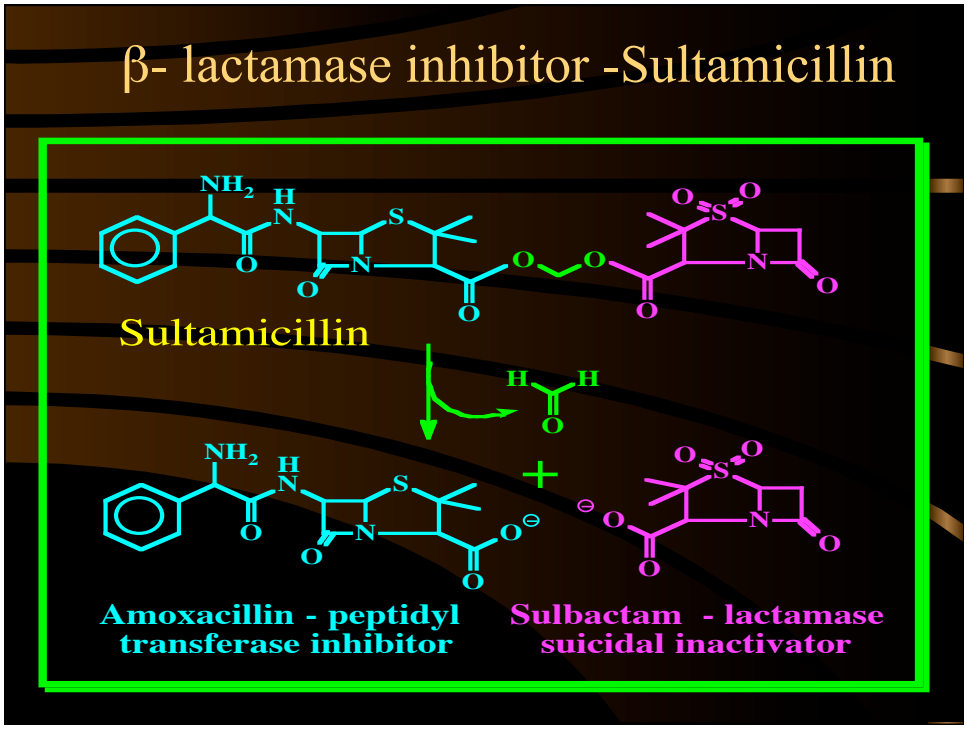


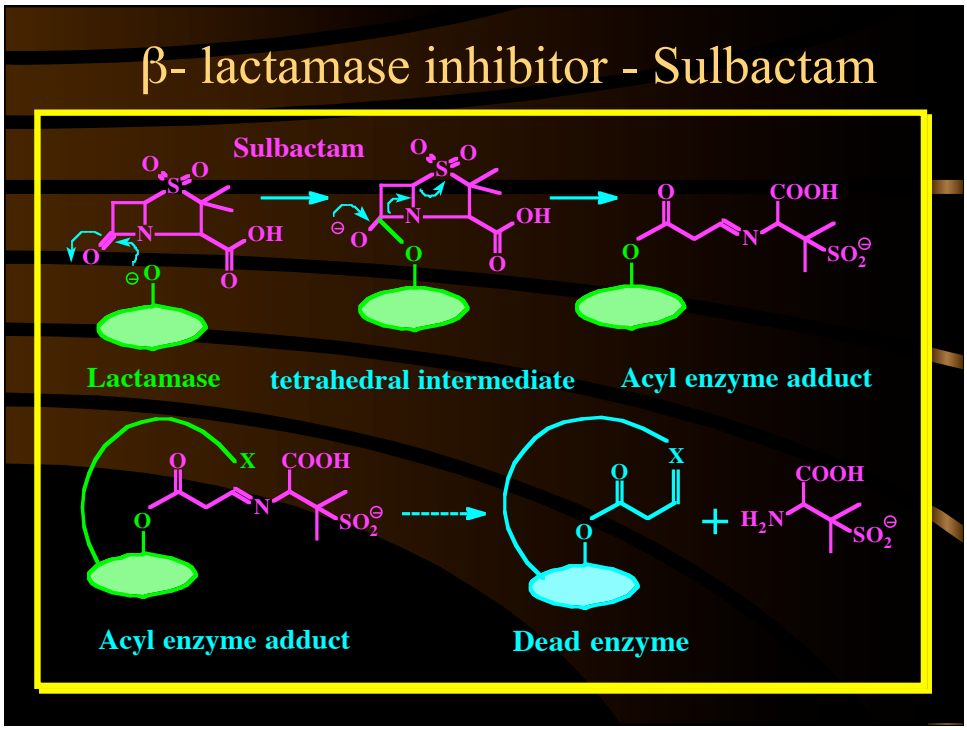
Stoichiometric titrant for chymotrypsin

Isatoic anhydride

Anthranilyl enyzme resistant to hydrolysis

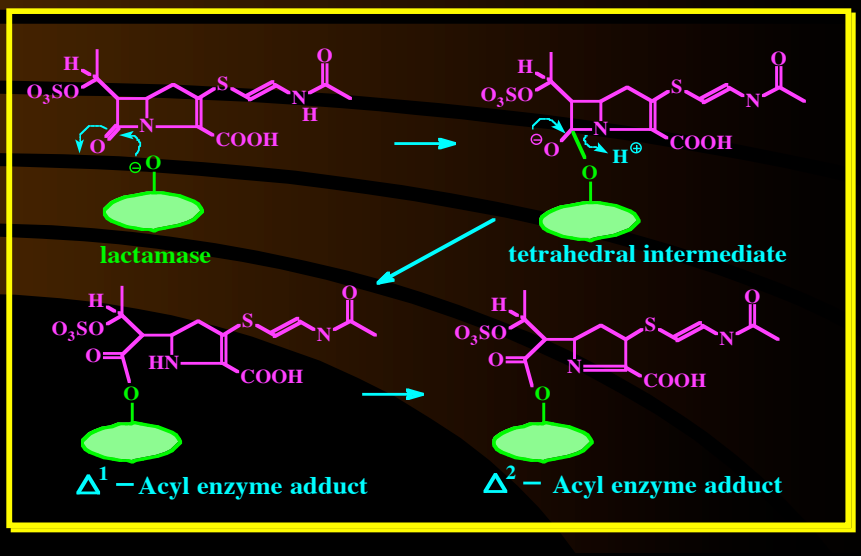



β- lactamase inhibitor -Sultamicillin
Sultamicillin
Amoxacillin - peptidyl transferase inhibitor
Sulbactam - lactamase suicidal inactivator


β- lactamase inhibitor - Sulbactam
Sulbactam
Lactamase
tetrahedral intermediate
Acyl enzyme adduct
Acyl enzyme adduct
Dead enzyme

β- lactamase inhibitor - Olivanate

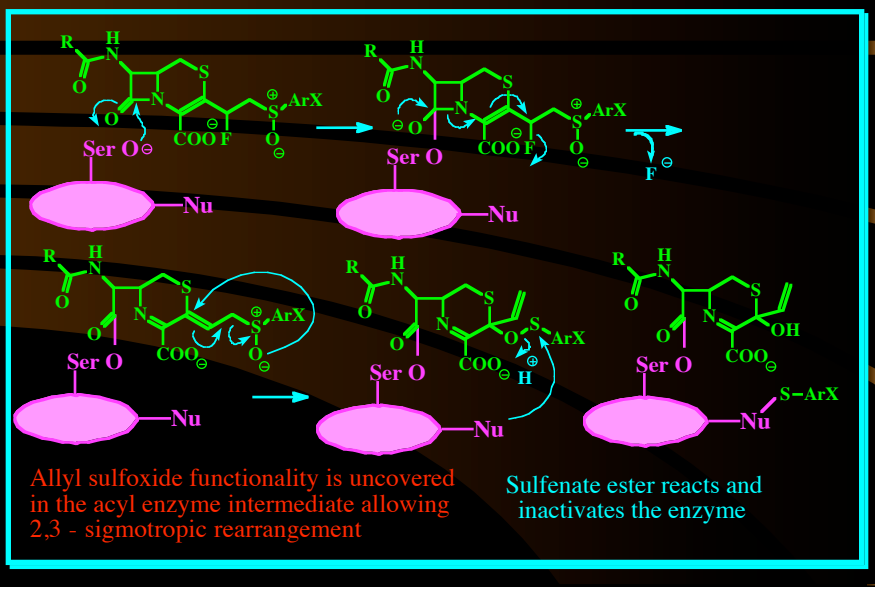
Both natural and synthetic carbapenems containing pyrroline ring inactivate lactamase by the following mechanism.



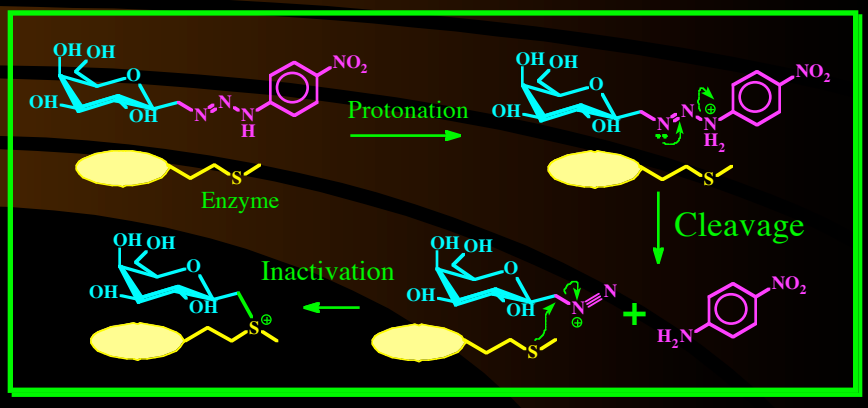
Lactamase suicidal substrate

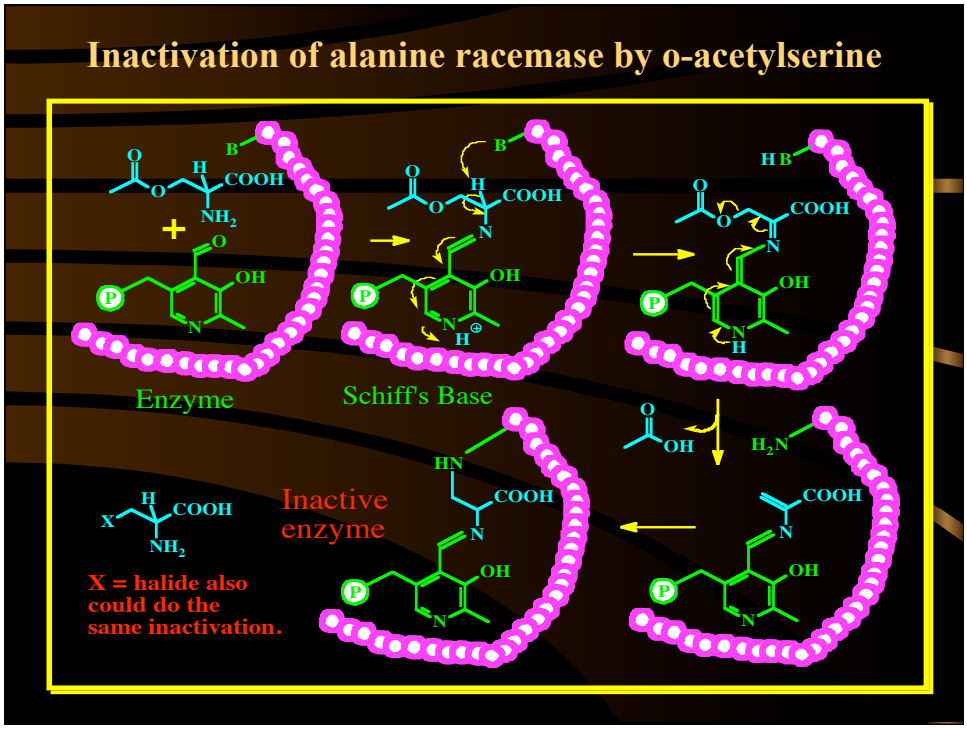
One mole of acetyl methylene penicillanic acid kills lactamase with IC_{50} value of 1.4×10^{-9} M.

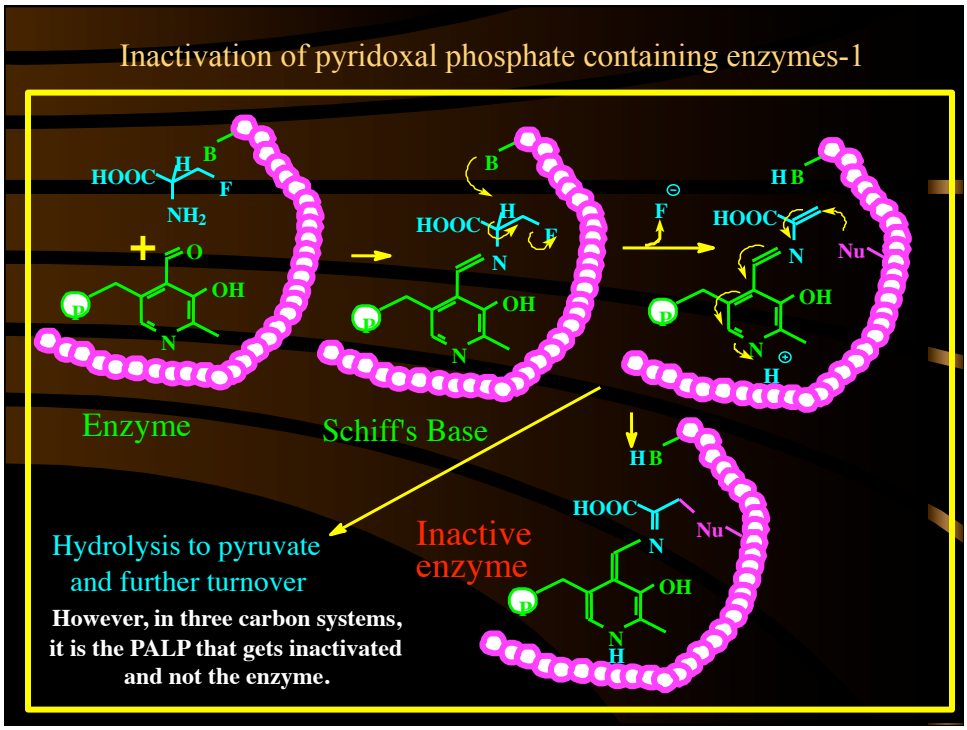
Cephalosporinase inhibitor based on 3'-exo substituent on cephalosporin hydrolysis

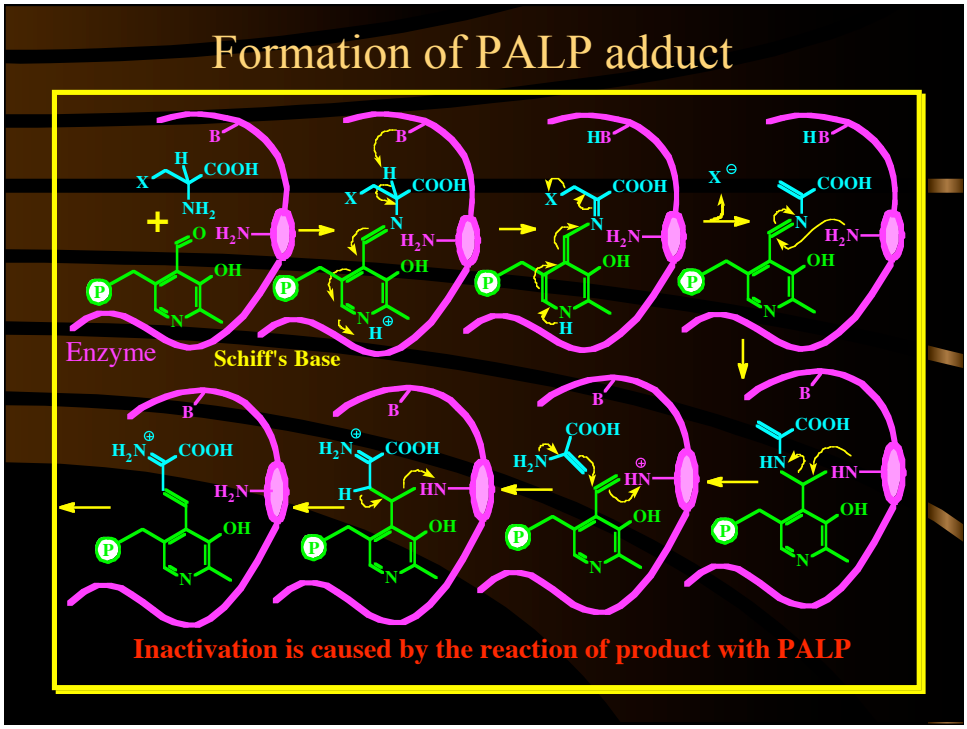


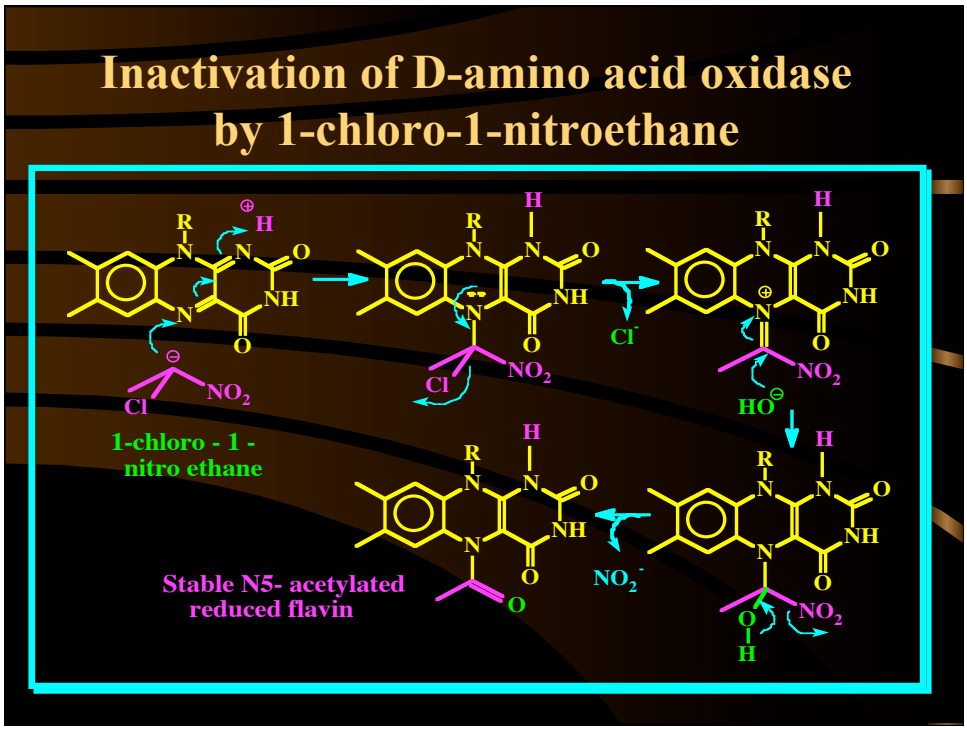
β-glycosidase inhibitor : β - D-galacto pyranosyl-*p*-nitrophenyltriazine in activated a β- glycosidase by adding on to the methionine at 500 position.

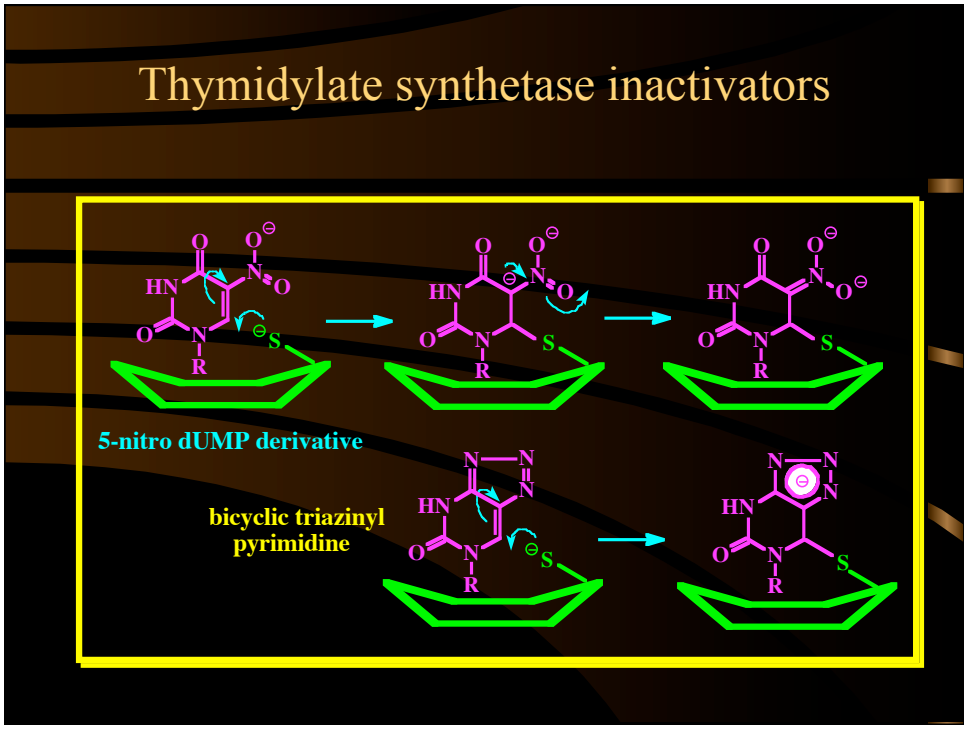


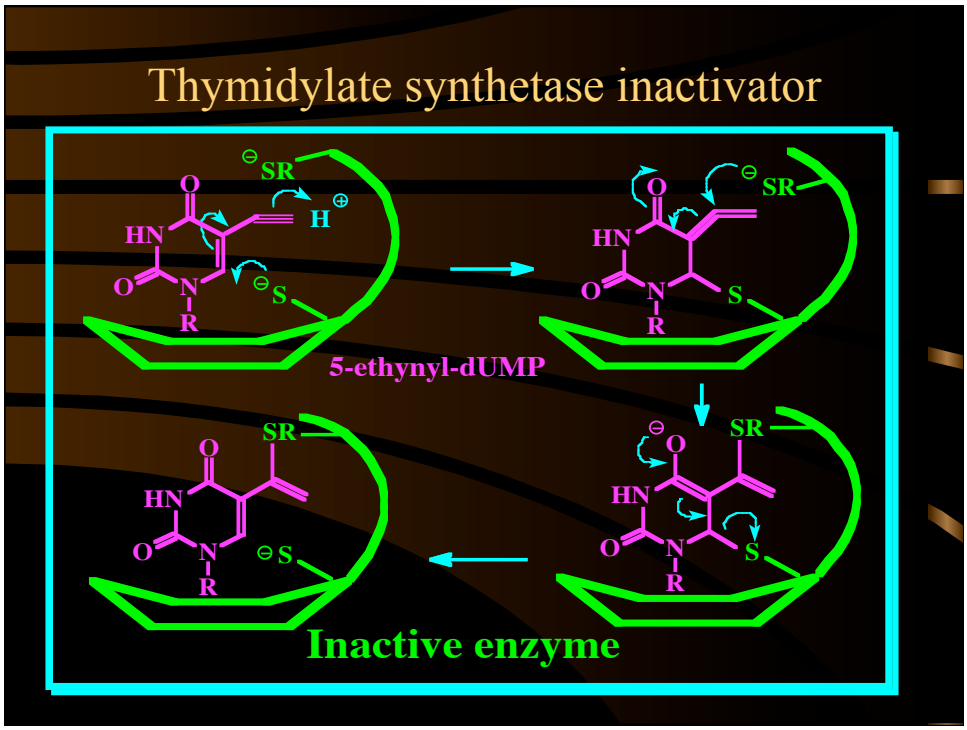

Inactivation of alanine racemase by o-acetylserine
Enzyme
Schiff's Base
Inactive enzyme
X = halide also could do the same inactivation.


Inactivation of pyridoxal phosphate containing enzymes-1
Enzyme
Schiff's Base
Hydrolysis to pyruvate and further turnover
Inactive enzyme
However, in three carbon systems, it is the PALP that gets inactivated and not the enzyme.

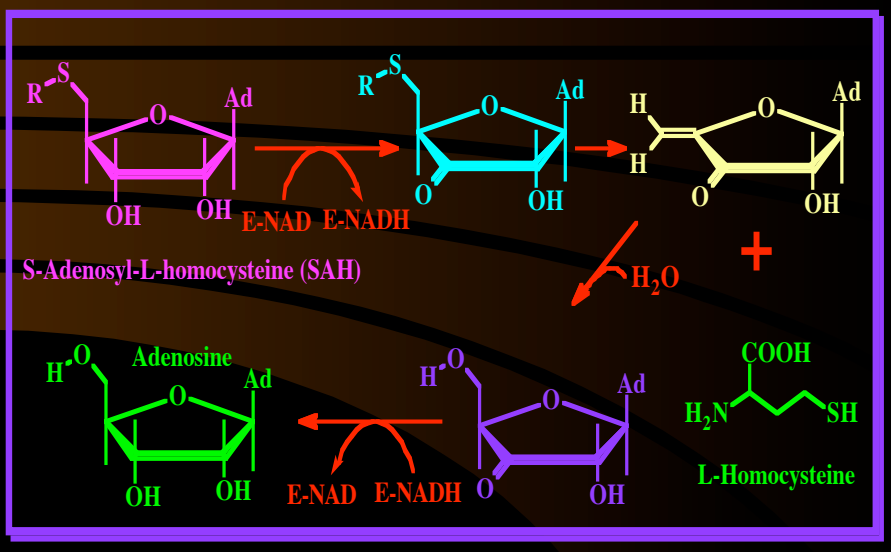

Formation of PALP adduct
Enzyme
Schiff's Base
Inactivation is caused by the reaction of product with PALP


Inactivation of D-amino acid oxidase
by 1-chloro-1-nitroethane
1-chloro - 1 -
nitro ethane
Stable N5- acetylated
reduced flavin

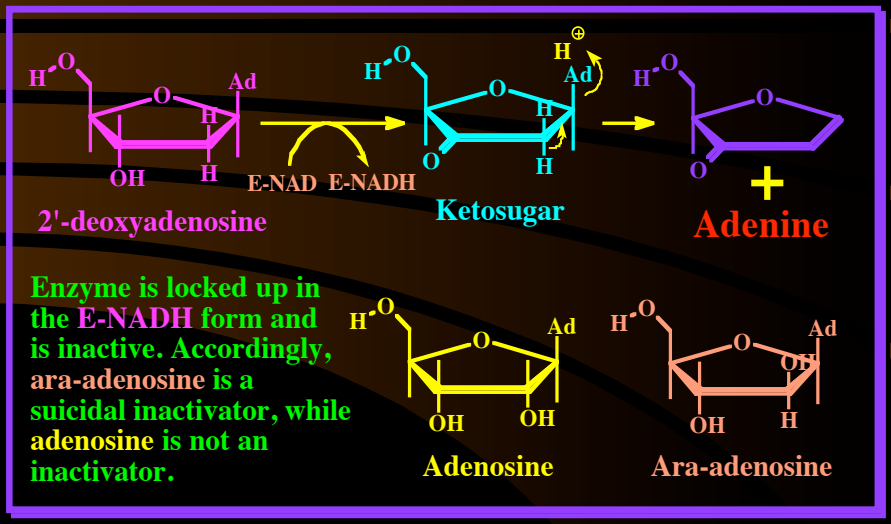

Thymidylate synthetase inactivators
5-nitro dUMP derivative
bicyclic triazinyl pyrimidine

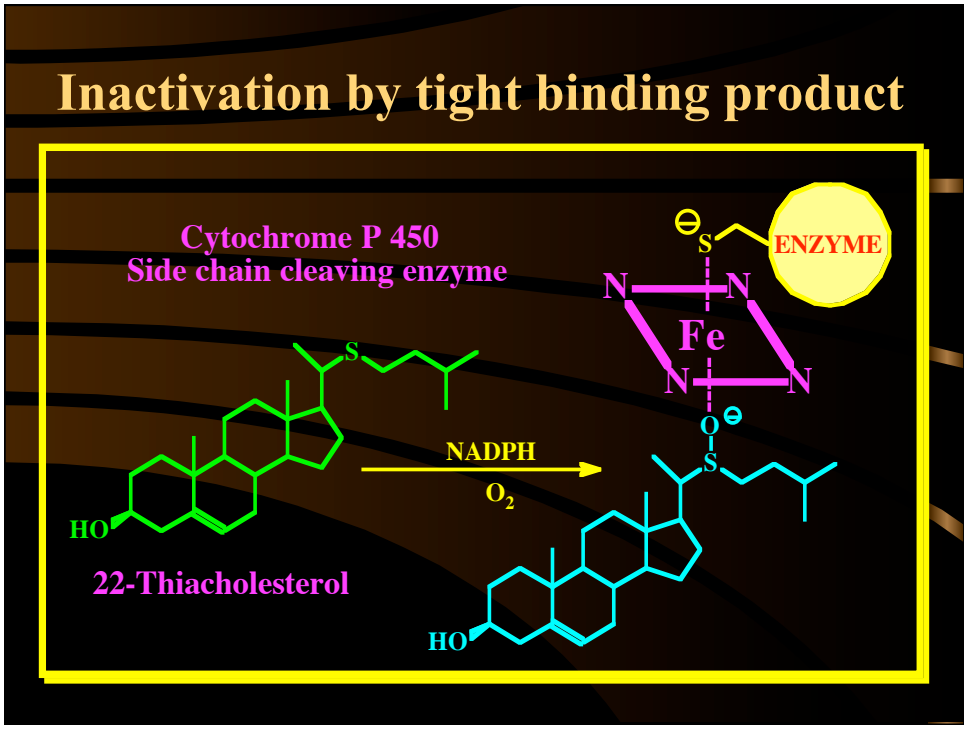

Thymidylate synthetase inactivator
5-ethynyl-dUMP
Inactive enzyme

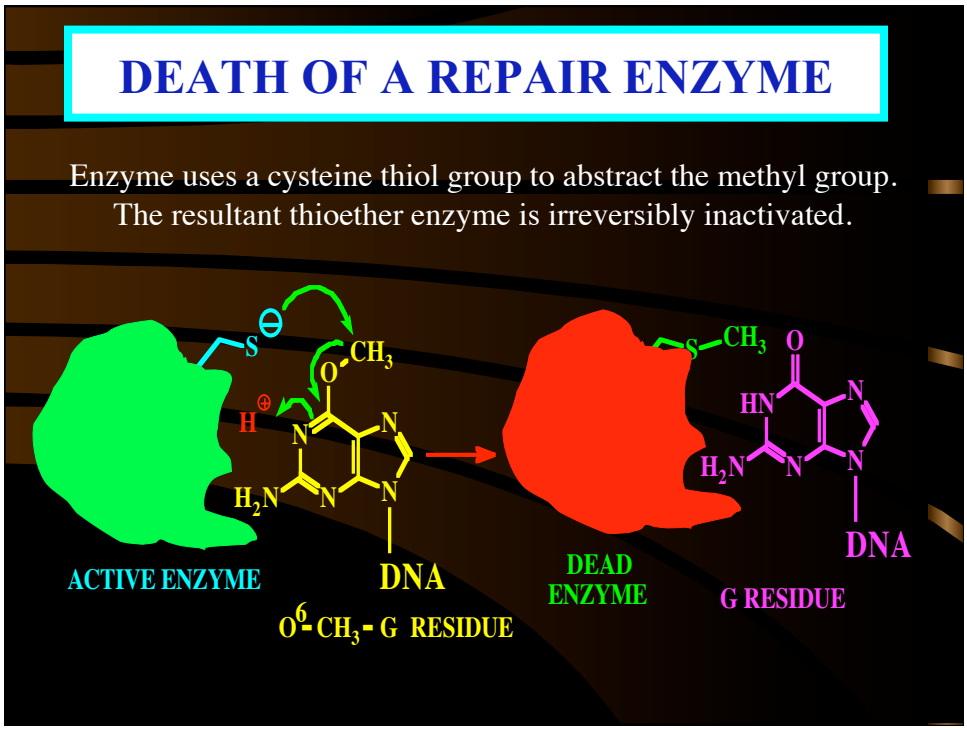
Reaction catalyzed by S-Adenosyl-L-homocysteinase

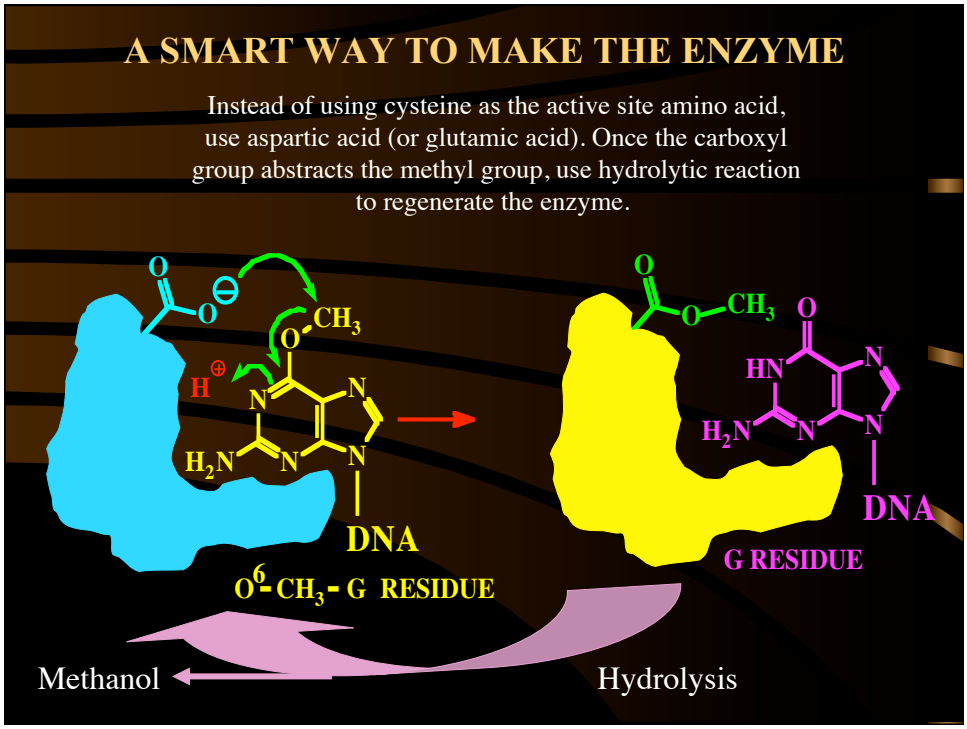


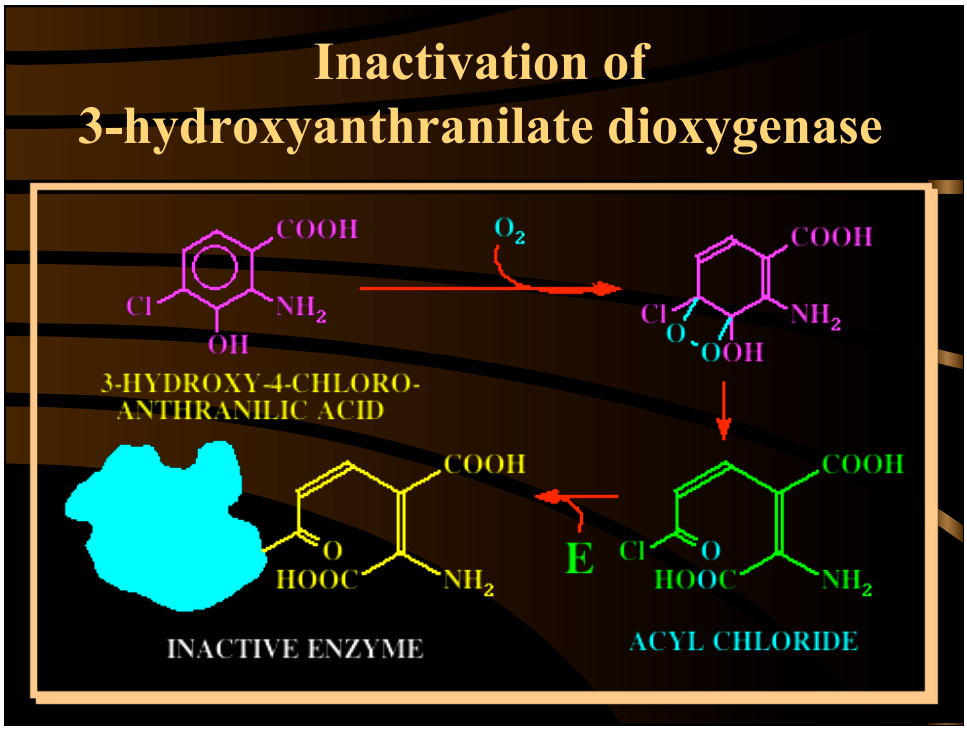
•Inactivation of S-Adenosyl-L-homocysteinase by 2'-deoxyadenosine



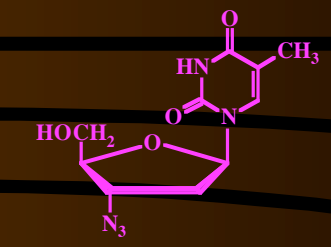

Inactivation by tight binding product
Cytochrome P 450
Side chain cleaving enzyme
ENZYME
Fe
NADPH
O_2
HO
22-Thiacholesterol
HO


DEATH OF A REPAIR ENZYME
Enzyme uses a cysteine thiol group to abstract the methyl group.
The resultant thioether enzyme is irreversibly inactivated.
ACTIVE ENZYME
DNA
O^6-CH_3-G RESIDUE
DEAD ENZYME
G RESIDUE
DNA


A SMART WAY TO MAKE THE ENZYME
Instead of using cysteine as the active site amino acid, use aspartic acid (or glutamic acid). Once the carboxyl group abstracts the methyl group, use hydrolytic reaction to regenerate the enzyme.
DNA
O6-CH3-G RESIDUE
G RESIDUE
Methanol
Hydrolysis


Inactivation of
3-hydroxyanthranilate dioxygenase
3-HYDROXY-4-CHLORO-ANTHRANILIC ACID
INACTIVE ENZYME
ACYL CHLORIDE

AZT INHIBITS DNA POLYMERASE BY CHAIN TERMINATION MECHANISM



3'-azido-3'deoxythymidine (AZT)

AZT gets converted into AZT triphosphate and is incorporated into the growing chain of DNA. But it lacks the 3' hydroxyl to allow further growth of the chain. Hence chain termination occurs.