3.1. Introduction to Enzyme Catalysis and Kinetics

3.1.1. Catalysis

Catalysis is the change in rate of a Chemical/Biochemical reaction due to the change in activation energy of that reaction by the involvement of a catalyst. A catalyst by nature and its definition, is not consumed by the reaction unlike other reagents participate in the chemical reaction. A catalyst may participate in multiple chemical transformations. Acceleration of the reaction rate is called positive catalysis and the catalysts speed up the same is called positive catalyst. On the other hand, substances that interact with catalysts to slow the reaction are called inhibitors or negative catalysts and the process is known as negative catalysis. Substances that increase the activity of catalysts are called promoters, and substances that deactivate catalysts activity are called catalytic poisons.

A catalyst generally brings down the energy of the activated state and thus the catalytic reactions have a lower rate-limiting free energy of activation than the corresponding unanalyzed reaction, resulting in higher reaction rate at the same temperature. However, the mechanistic explanation of catalysis is complex. Catalysts affect the reaction environment favorably by binding to the reagents to polarize bonds.

3.1.2. Catalysis and Reaction Energetic

In general, catalysts work by providing an alternative pathway which involves a different transition state of lower activation energy compared to an unanalyzed reaction. Consequently, more molecular collisions have the energy needed to reach the transition state. Hence, catalysts enable reactions that would otherwise be blocked or slowed by a kinetic barrier. The catalyst can increase reaction rate. It can enhance selectivity, or the reaction proceeds at lower temperatures in presence of a catalyst.

In the catalyzed elementary reaction, catalysts do not change the extent of a reaction. Thus, they have no effect on the chemical equilibrium of a reaction because the rate of both the forward and the reverse reaction are affected equally. This fact is a consequence of the second law of thermodynamics. If a catalyst changes the equilibrium, then it must be consumed as the reaction proceeds, and thus it is also a reactant. The catalyst stabilizes the transition state more than it stabilizes the starting material. Therefore, it decreases the kinetic barrier by decreasing the *difference* in energy between starting material and transition state (**Figure 3.1**).

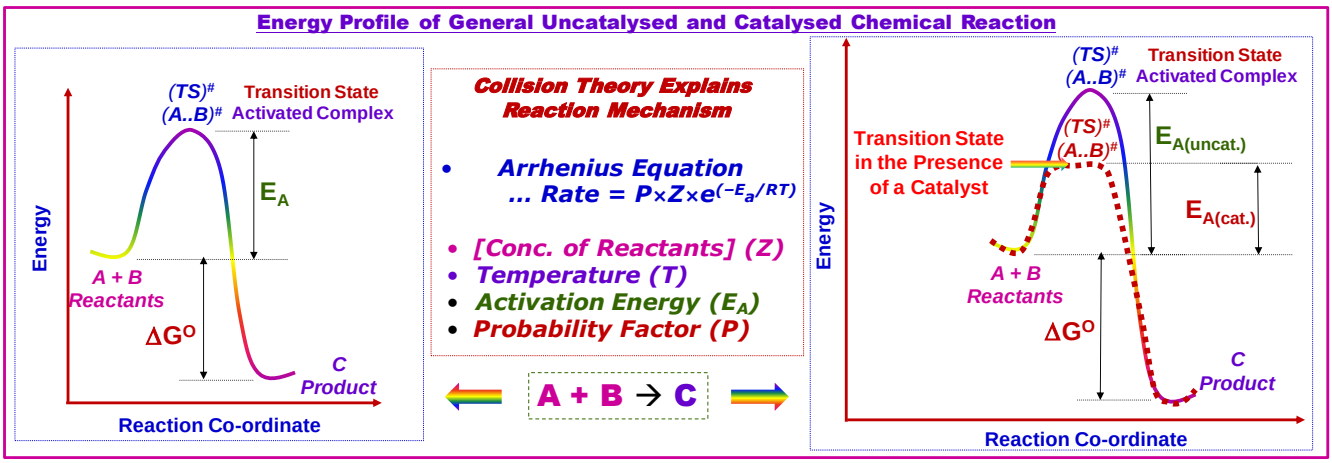


Figure 3.1: Potential energy diagram showing the effect of a catalyst in a hypothetical exothermic chemical reaction A + B to give product C. The presence of the catalyst opens a different reaction pathway with lower activation energy.

3.1.3. Types of Catalysis

Catalysts can be either heterogeneous or homogeneous, depending on whether a catalyst exists in the same phase as the substrate. Following are the main types of catalysts.

- **Heterogeneous catalysts**
- **Homogeneous catalysts**
- **Electro catalysts**
- **Organocatalysis**
- **Biocatalysts (enzymes)**

3.2. Biocatalysis: The Enzyme as a Catalyst

3.2.1. Definition

Biocatalysis is the use of natural catalysts, such as a catalytic protein which is most of the time referred to as an enzyme, to perform chemical transformations on organic compounds or biochemical reaction inside the living cells. Catalysis of biochemical reactions in the cell is vital due to the very low reaction rates of the unanalyzed reactions.

3.2.2. Brief History of Enzymes

- In, 1833, the first enzyme was isolated from aqueous extract of malt added ethanol. The heat-labile precipitate that is now known as amylase was utilized to hydrolyze starch to soluble sugar.
- In 1878, Kühne coined the term *enzyme* which means “in yeast”.
- In 1898, Duclaux proposed that all enzymes should have suffix “ase”.
- Thus, Enzymes was defined as natural proteins capable of catalyzing chemical reactions.

- Enzymes have molecular weights of several thousand to several million, yet it can catalyze transformations on molecules as small as carbon dioxide and nitrogen.
- Enzymes function by lowering transition-state energies and energetic intermediates and by raising the ground-state energy.
- First enzyme recognized as protein was jack bean urease, crystallized in 1926 by James B. Sumner of Cornell University. He later, in 1946, received the Nobel Prize for his work with the enzyme urease.
- Urease is an enzyme that catalyzes the conversion of urea to ammonia and carbon dioxide. Certain bacteria that convert urea to ammonia as part of the nitrogen cycle contain this enzyme.

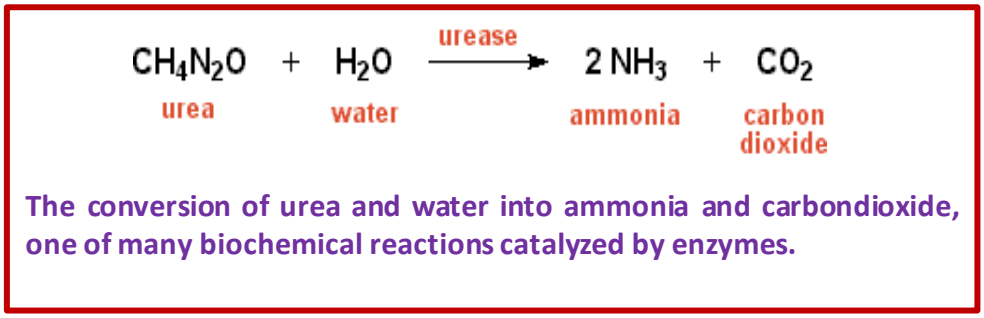


Figure 3.2: Ureas enzyme catalyzed conversion of urea to ammonia

- Almost all Enzymes are proteins. Their ability to catalyze reactions is attributable to their primary, secondary, tertiary, and quaternary structures.
- As catalysts, enzymes are both efficient and highly specific for a particular chemical reaction.
- Cofactors are involved in reactions along with enzyme where molecules are oxidized, reduced, rearranged or connected.

3.2.3. Characteristic of Enzyme Catalysis

- Almost all biochemical processes are catalyzed by enzymes.
- As almost all Enzymes are proteins, their ability to catalyze reactions is attributable to their primary, secondary, tertiary, and quaternary structures.
- Enzymes have a high degree of specificity for their substrates.
- Enzymes accelerate chemical reactions tremendously.
- Enzymes can function in aqueous solution under mild conditions, which are unlike the conditions that are frequently needed in organic chemistry.
- Enzymes are effective in minute amounts because they are not used up in the reaction that they catalyze.
- Enzymes do not affect the direction of the reaction but make the reaction reach equilibrium sooner.
- Both synthesis and decomposition of molecules in a living system normally proceed too slowly to be useful to metabolic survival. However,

the presence and activity of enzymes speed up those to support life activities of cellular metabolism.

- Enzymes make up a substantial portion of the total protein of the cell. A typical cell contains about 3000 different kinds of enzyme molecules and many copies of each kind.
- Within a cell, chemical reactions take place within a narrow temperature and pH range. This is possible because enzymes generally lower the activation energy of a reaction through a variety of mechanisms.
- **Enzymes necessarily are very large, because,**
  (a) Most effective binding of substrate results from close packing of atoms within enzymes.
  (b) Remainder of enzyme outside active site is required to maintain integrity of the active site.
  (c) May serve to channel the substrate into the active site.
  (d) Active site aligns the orbitals of substrates and catalytic groups on the enzyme optimally for conversion to the transition-state structure--called *orbital steering.*

3.2.3. How Enzymes work?

- Many reactions in biochemistry are spontaneous, i.e. they are thermodynamically favorable (□G < 0).
- The oxidation of glucose to produce carbon dioxide and water is thermodynamically favorable (□G°= -2870 kJ/mol). However, a jar of sugar in water is highly stable in the absence of microbial contamination.
- Biological reactions are almost always under kinetic control. Thus, a given amount of energy must be put into the system (energy of activation) in order for energy to be released from biochemical reactions. The rate of a reaction is related exponentially to the energy of activation.
- Two ways to effect the reaction are- i) raising the free energy of the substrates, or ii) decreasing the energy of activation for the reaction.
- Enzymes catalyze reactions by lowering the activation energy barrier.

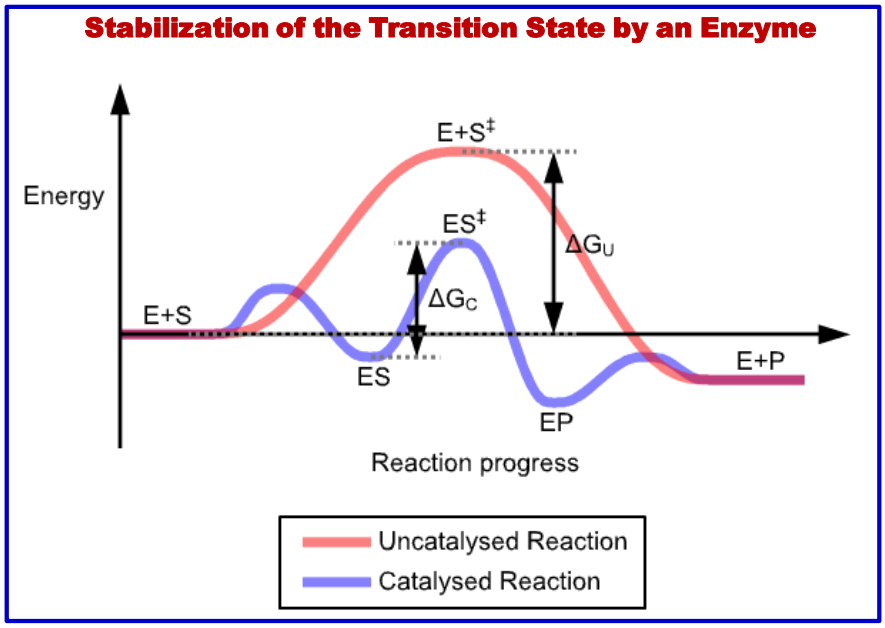


Figure 3.3: Energetics of stabilization of T.S. by an enzyme.

3.3. Advantages and Important Stereochemical Features of Enzyme Catalysis:

1. Enzymes are natural proteins, available from renewable resources.

2. Enzyme catalysis need: Mild Reaction Conditions – 37^{o} C, near neutral pH and std. pressure.

3. Enzymes are Biodegradable – waste management problems reduced.

4. They are highly specific.

5. Enzymes display three major types of selectivities:

   - Chemoselectivity: Enzyme acts on a single type of functional group, other sensitive functionalities, which would normally react to a certain extent under chemical catalysis, survive. As a result, enzyme catalyzed reactions tend to be cleaner.

   - Regioselectivity and diastereoselectivity: Enzyme, out of their complex three-dimensional structure, can distinguish between functional groups situated in different regions of the substrate molecule.

- Enantioselectivity: Enzymes are protein, made up of L-amino acids→, so, enzymes are chiral catalysts. So, any type of chirality present in the substrate molecule is recognized in the enzyme-substrate complex. Thus a prochiral substrate may be transformed into an optically active product and both enantiomers of a racemic substrate may react at different rates.

6. Because of enantioselective nature, synthetic chemists have become interested in enzyme catalyzed enantioselective transformation.

7. They are environmentally acceptable (‘GREEN’), as they are completely degraded in the environment.

3.4. Hypotheses for Enzyme Catalyzed Reactions

- Many different hypotheses proposed for how enzymes catalyze reactions. However, the common link of all hypotheses is- enzyme-catalyzed reaction always initiated by the formation of an *enzyme-substrate* (or *E•S*) *complex* in a small cavity called the active site.

Hypotheses for Enzyme Catalyzed Reactions

Lock-and-key Hypothesis

•**Emil Fischer, in 1894, proposed that the enzyme is the lock into which the substrate (the key) fits.**

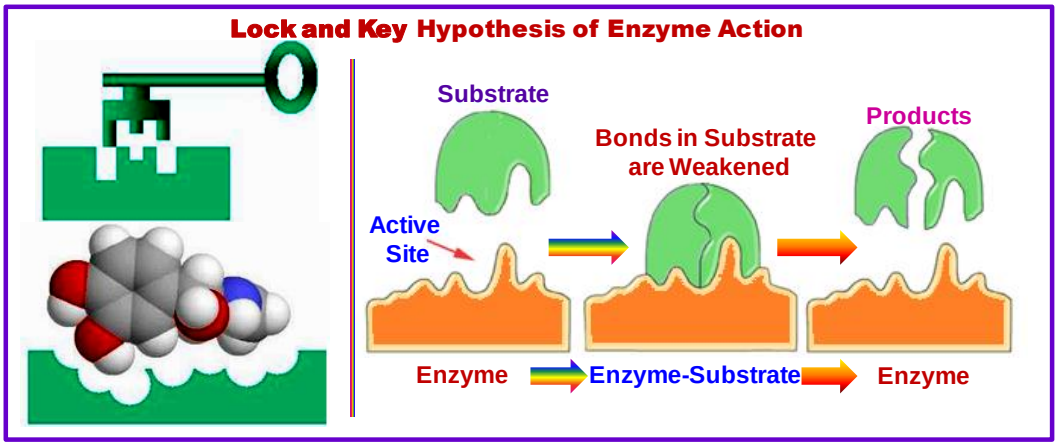
- **Every 'lock' has its own 'key'**
- **If the 'key' is not precise, the 'lock' does not open**
- **The 'drug' is the key that has to fit the target specifically and productively**

•**However, it does not rationalize certain observed phenomena:**

(a) Compounds having *less* bulky substituents often fail to be substrates.

(b) Some compounds with *more* bulky substituents bind *more* tightly.

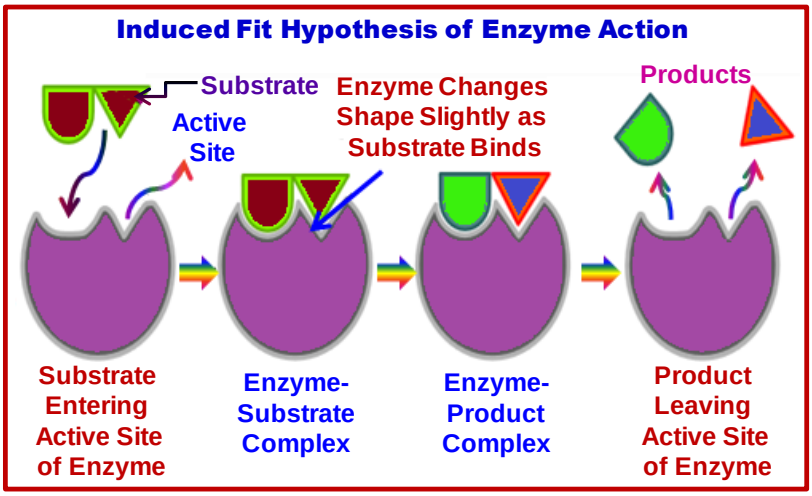
(c) Some enzymes that catalyze reactions between two substrates do not bind one substrate until the other one is bound.



Hypotheses for Enzyme Catalyzed Reactions

Induced-fit Hypothesis

- **Koshland proposed this theory in1958.**
- **Substrate binding to an enzyme → induce a *conformational change* in the enzyme → thus, enzyme changes from a low catalytic form to a high catalytic form.**
- **Induced-fit hypothesis requires a flexible active site.**
- **Flexible active site: Concept proposed by Pauling (1946): Enzyme is a flexible template that is most complementary to substrates at the transition state rather than at the ground state → thus, the substrate does not bind most effectively in the E•S complex → transition state is stabilised.**
- ***Transition-state stabilization* results in rate enhancement.**
- **Active site made up of only a dozen or so amino acid residues out of which only two or three may be involved directly in substrate binding and/or catalysis.**



3.5. Strategies of Enzyme Catalysis

- **Acid-base catalysis**
  - A molecule other than water plays the role of a proton donor or acceptor.
- **Covalent catalysis**
  - The active site contains a reactive group, usually a powerful nucleophile that becomes temporarily covalently modified in the course of catalysis.
- **Metal ion catalysis**
  - Metal ions can serve as electrophilic catalyst, stabilizing negative charge on a reaction intermediate.

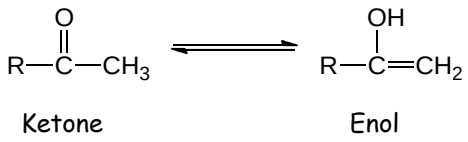
- **Catalysis by approximation**
    - In reactions that include two substrates, the rate is enhanced by bringing the two substrates together in a proper orientation.
- **Proximity and orientation effects**
- **Preferential binding (stabilization) of the transition state**

3.5.1. Acid-Base Catalysis: Catalysis Involving Proton Donors or Acceptors

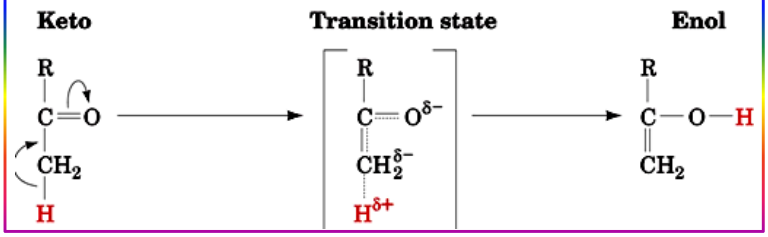
- Proton donors and acceptors, i.e. acids and bases, may donate and accept protons in order to stabilize developing charges in the transition state.
- This has the effect of activating nucleophile and electrophile groups, or stabilizing leaving groups. Histidine is often the residue involved in these acid-base reactions, since it has a pKa close to neutral pH and can therefore both accept and donate protons.
- Many reaction mechanisms involving acid/base catalysis assume a substantially altered pKa. This alteration of pKa is possible through the local environment of the residue.
- The pKa is can be modified significantly by the environment, to the extent that residues which are basic in solution may act as proton donors, and vice versa.
- The catalytic effect of the above example is mainly associated with the reduction of the pKa of the oxy anion and the increase in the pKa of the histidine, while the proton transfer from the serine to the histidine is not catalyzed significantly, since it is not the rate determining step.

Acid-Base Catalysis in Enezyme

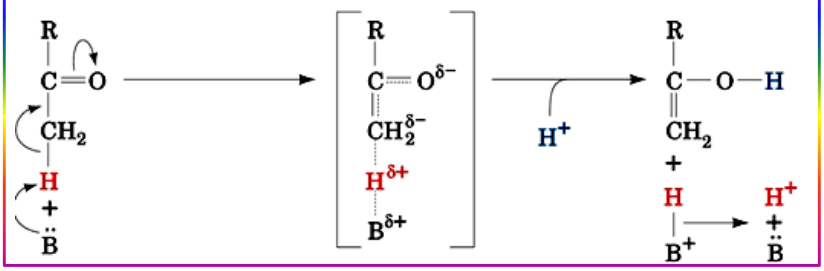
Keto-Enol Tautomerization



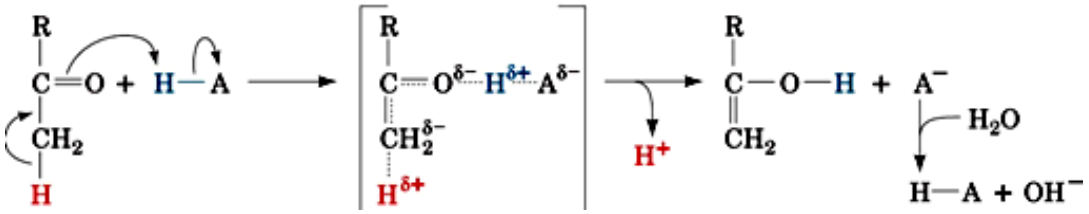
The Uncatalyzed Keto-Enol Tautomerization



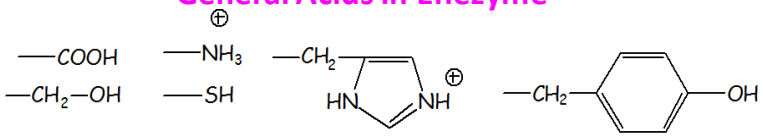
General Base Catalysis Keto-Enol Tautomerization



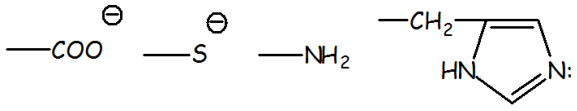
General Acid Catalysed Keto-Enol Tautomerization



General Acids In Enezyme



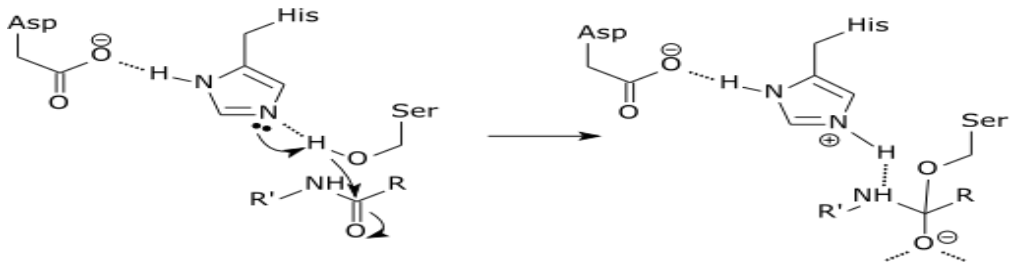
General Bases in Enezyme



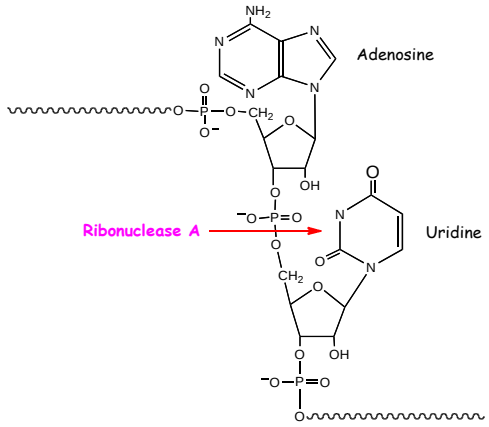
Acid-Base Catalysis in Enezyme

Catalytic Mechanism of Serine Protease

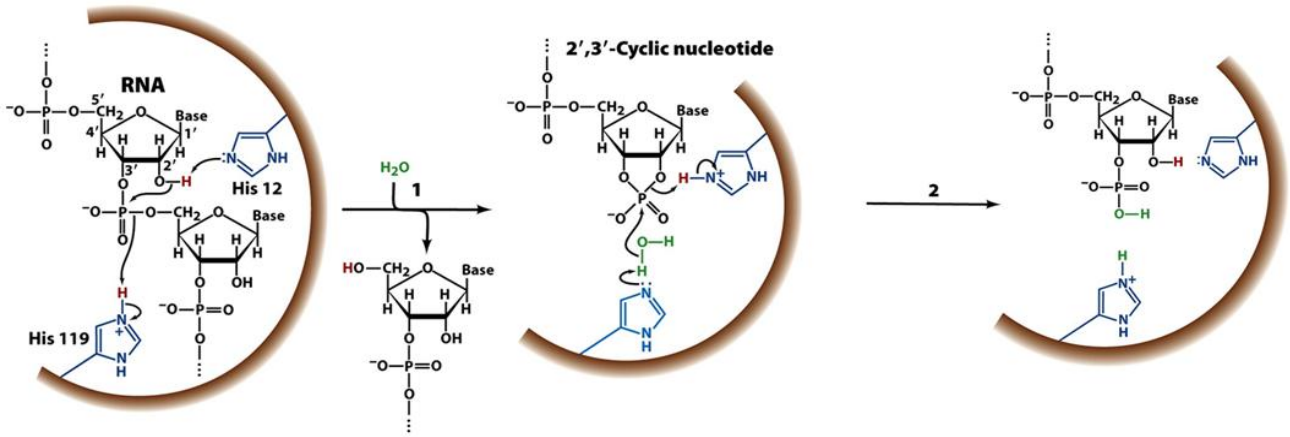
The initial step of the catalysis of serine protease involves the histidine of the active site accepting a proton from the serine residue. This prepares the serine as a nucleophile to attack the amide bond of the substrate. This mechanism includes donation of a proton from serine (a base, pKa 14) to histidine (an acid, pKa 6), made possible due to the local environment of the bases.



Ribonuclease A



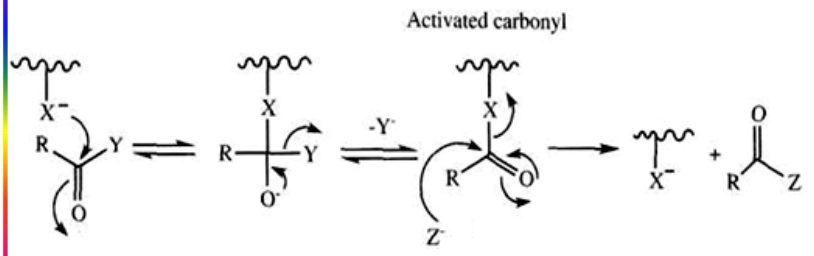
Mechanism of RNase A



3.5.2. Covalent Catalysis: Catalysis Involving Covalent Bond Formation

Covalent Catalysis

- The principle advantage of using an active site residue instead of water directly is that formation of covalent linkage leads to unimolecular reaction, which is entropically favored over the bimolecular reaction.



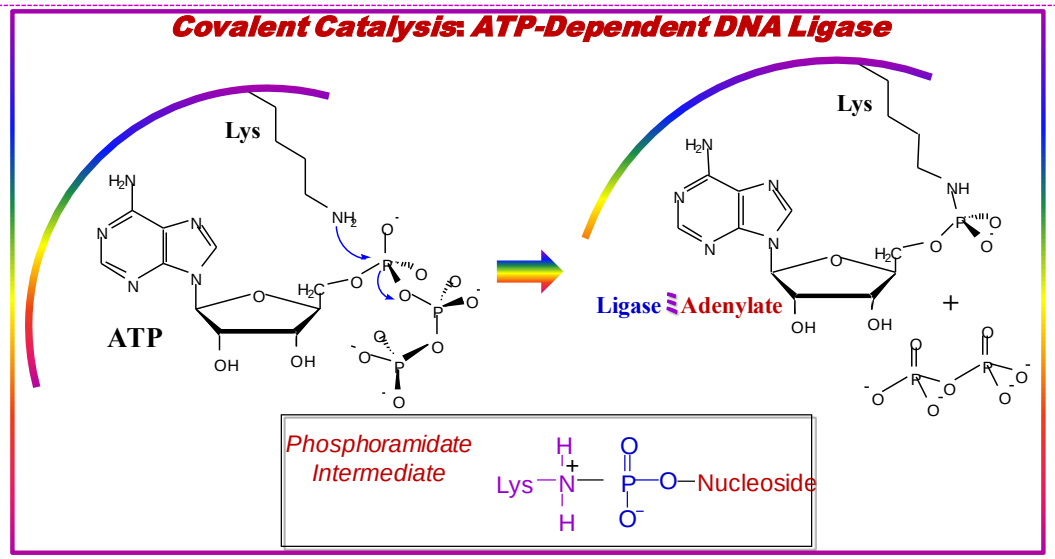
- Y should be a better leaving group than X.
- X is a better attacking group then Z.
- Covalent intermediate should be more reactive than substrate.

- Enzyme that utilize covalent catalysis are generally two step process: formation and breakdown of covalent intermediate rather than catalysis of the single reaction directly.

The Nucleophilic Groups in Enezymes

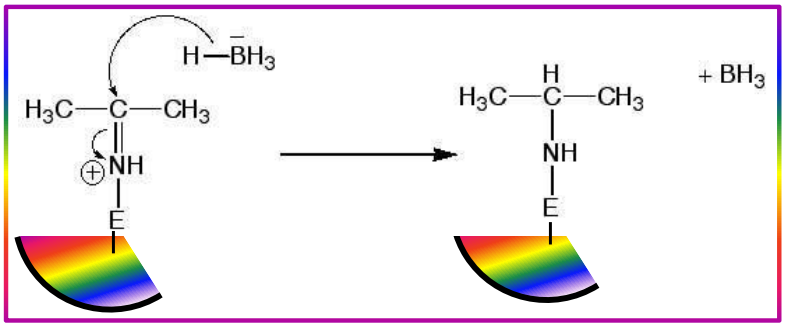
Aspartate	caboxylates	R = $-CH_2-C(=O)-O^-$	Tyrosine	hydroxyl-	R = $-CH_2-C_6H_4-OH$
Glutamates	caboxylates	R = $-CH_2-CH_2-C(=O)-O^-$	Lysine	amino-	R = $-CH_2CH_2CH_2CH_2NH_3^+$
Cystine	thiol-	R = $-CH_2SH$	Histadine	imidazolyl-	R = $-CH_2-C_3H_3N_2$
Serine	hydroxyl-	R = $-CH_2OH$			

Covalent Catalysis

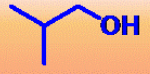
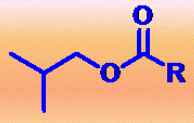
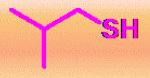
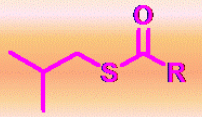
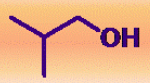
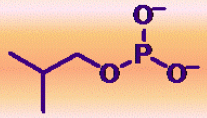
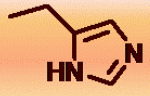
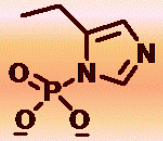
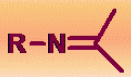


Covalent catalysis: Schiff Base Formation

- Enzymes that form Schiff base intermediates are typically irreversibly inhibited by the addition of sodium borohydride ($Na^+ BH_4^-$).
- Borohydride *reduces* the Schiff base and "traps" the intermediate such that it can no longer be hydrolyzed to release the product from the enzyme.
- This is often used as evidence for a mechanism involving an enzyme-linked Schiff base intermediate.

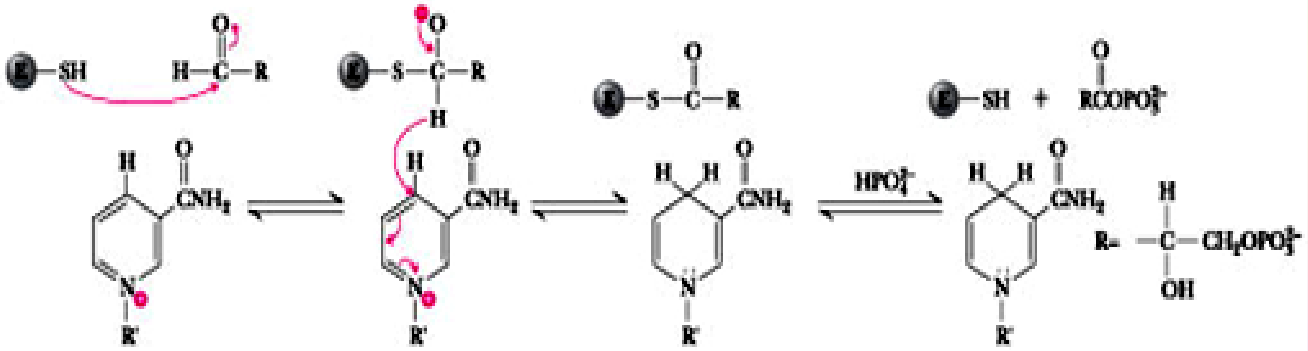


Enzymes That Form Covalent Intermediates

Enzymes	Reacting Group	Covalent Intermediate
1. Chymotrypsin Elastase Esterases Subtilisin Thrombin Trypsin		
2. Glyceraldehyde-3-phosphate dehydrogenase Papain		
3. Alkaline phosphatase Phosphoglucomutase		
4. Phosphoglycerate mutase Succinyl-CoA synthetase		
5. Aldolase Decarboxylases Pyridoxal phosphate-dependent enzymes		

Covalent Intermediate in the Gglyceraldehyde-3-Phosphate Dehydrogenase Reaction

The reaction mechanism involves nucleophilic attack by -SH on the substrate glyceraldehyde-3-phosphate to form a covalent acylcysteine (or hemithioacetal) intermediate. Hydride transfer to NAD^+ generates a thioester intermediate. Nucleophilic attack by phosphate yields the desired mixed carboxylic-phosphoric anhydride product, 1,3-bisphosphoglycerate.

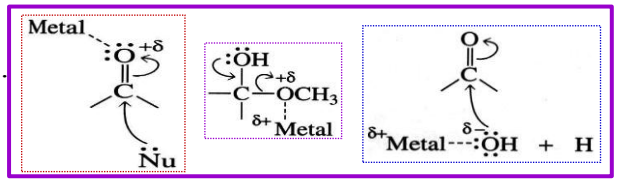


3.5.3. Metal Ions Catalysis

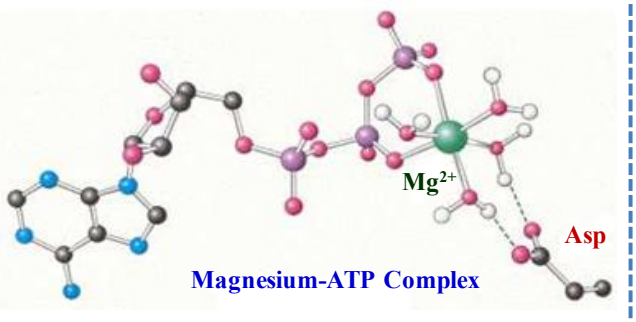
Metal Ions Catalysis

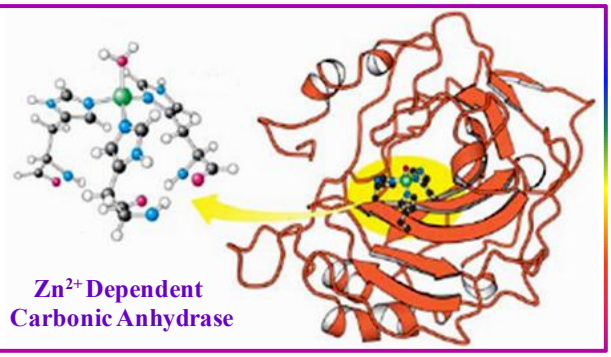
Metal ions can perform:

- Electrostatically stabilizing or shielding negative charges.
- Act much like a proton but can be present in high concentration at neutral pH and can have multiple positive charges.
- Act to bridge a substrate and nucleophilic group.
- Bind to substrates to insure proper orientation.

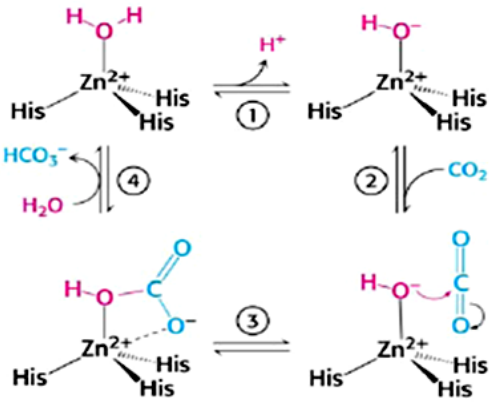


- Participate in oxidation/reduction mechanisms through change of oxidation state.





Mechanism of Carbonic Anhydrase



- Zinc facilitates the release of a proton from a water molecule.
- CO2 binds to the enzyme active site, positioned to react with hydroxide ion.
- The hydroxide ion attack CO2, forming bicarbonate ion.
- Catalytic sit is regenerated by release of bicarbonate and binding of another water molecule.

3.5.4. Enzyme Catalysis by Approximation

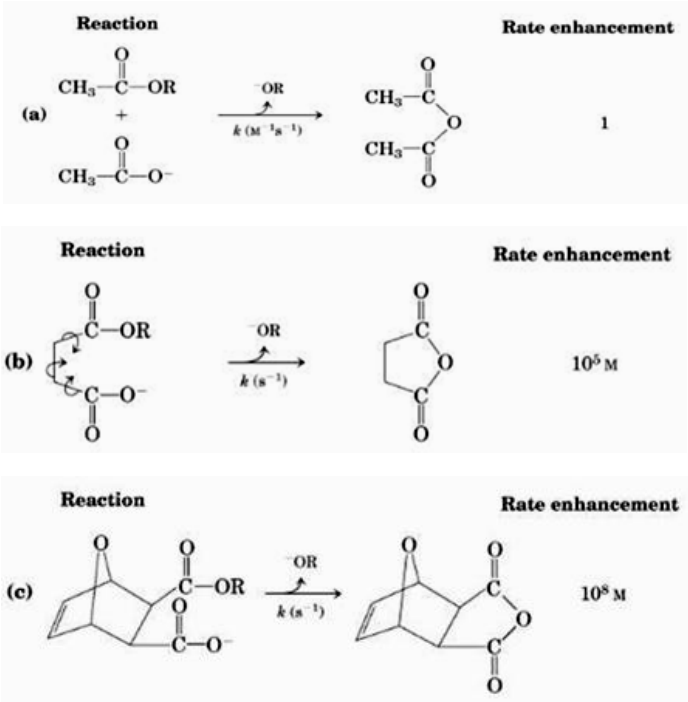
Enezyme Catalysis by Approximation

- Enzyme serves as a template to bind the substrates so that they are close to each other in the reaction center.
- They Bring substrate into contact with catalytic groups or other substrates.
- They Correct orientation for bond formation.
- They Freeze translational and rotational motion.

a) Bimolecular reaction (high activation energy, low rate).

b) Unimolecular reaction, rate enhanced by factor of 10^5 due to increased probability of collision/reaction of the 2 groups.

c) Constraint of structure to *orient* groups better (elimination of freedom of rotation around bonds between reactive groups), rate enhanced by *another* factor of 10^3, for 10^8 total rate enhancement over bimolecular reaction.



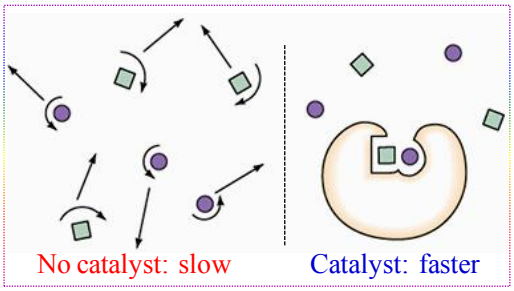
3.5.5. Proximity and Orientation Effects in Enzyme Catalysis

Proximity and Orientation Effects

- This increases the rate of the reaction as enzyme-substrate interactions align reactive chemical groups and hold them close together. This reduces the entropy of the reactants and thus makes reactions such as ligations or addition reactions more favorable, there is a reduction in the overall loss of entropy when two reactants become a single product.
- This effect is analogous to an effective increase in concentration of the reagents. The binding of the reagents to the enzyme gives the reaction intramolecular character, which gives a massive rate increase.

Rate of a reaction depends on

- Number of collisions
- Energy of molecules
- Orientation of molecules
- Reaction pathway (transition state)



Proximity:

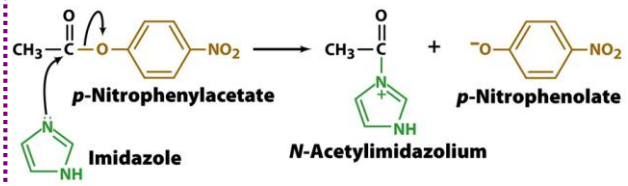
- Similar reactions will occur far faster if the reaction is intramolecular.
- Enezyme brings the two or more reactant closer in proximity to react.
- Brings substrate with their catalytic groups closer.
- Electrostatic catalysis:
  - transition state stabilization the charge distribution around the active sites guide polar substrates toward their binding site.
- Freezing out the molecular motions:transition state stabilization:
  - translational and rotational motions of their substrates and catalytic groupsrate enhancement up to ~10^7

Orientation:

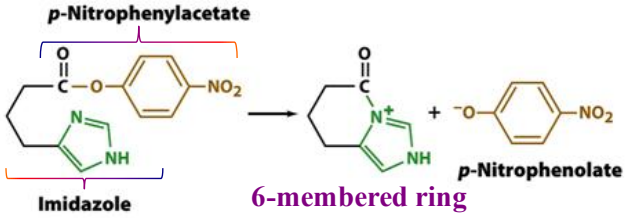
- Enzymes not only bring substrates and catalytic groups close together, they orient them in a manner suitable for catalysis as well. Comparison of the rates of reaction of the molecules shown makes it clear that the bulky methyl groups force an orientation on the alkyl carboxylate and the aromatic hydroxyl groups that makes them approximately 250 billion times more likely to react. Enzymes function similarly by placing catalytically functional groups (from the protein side chains or from another substrate) in the proper position for reaction.

Proximity Effect: Similar Intramolecular Reactions are Faster than Intermolecular

Intermolecular Biomolecular Reaction of Imidazole with *p*-Nitrophenylacetate



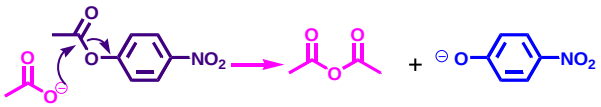
Intramolecular Biomolecular Reaction of Imidazole with *p*-Nitrophenylacetate



6-membered ring fused imidazolium

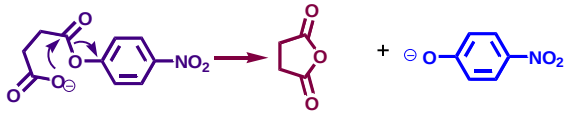
Intramolecular Rate = 24 x Intermolecular Rate

Intermolecular Biomolecular Reaction of Imidazole with *p*-Nitrophenylacetate



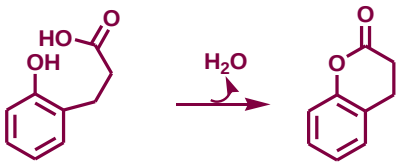
Rate = 4×10^{-6} $s^{-1}M^{-1}$

Intramolecular Biomolecular Reaction of Imidazole with *p*-Nitrophenylacetate

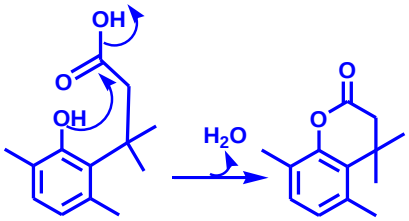


Rate = 0.8 s^{-1}

Orientation Effect: Steric Crowding and Rate Enhancement



Rate = 5.9×10^{-6}



Rate = 1.5×10^{6}

Rate Acceleration of 2.5×10^{11}

3.6. Thermodynamics and Mechanisms of Enzyme Catalysis

Enzymes can act in several ways, all of which lower activation free energy of the reaction ($\Delta G^{\ddagger}$):

- The activation energy is lowered by creating an environment in which the transition state is stabilized. The shape of a substrate is strained in shape upon binding the transition-state conformation of the substrate/product molecules → the enzyme distorts the bound substrate into their transition state form → thus, the amount of energy required to complete the transition is reduced.
- Enzyme may Lower the energy of the transition state without distorting the substrate by creating an opposite charge distribution to that of the transition state.
- Enzyme provides an alternative pathway. For example, temporarily reacting with the substrate to form an intermediate ES complex, which would be impossible in the absence of the enzyme.
- Bring the substrates together in the correct orientation to react → thus reducing the reaction entropy change by bringing.
- Increases in temperatures speed up reactions. Thus, temperature increases help the enzyme function and develop the end product even faster. However, if heated too much, the enzyme becomes denatured.

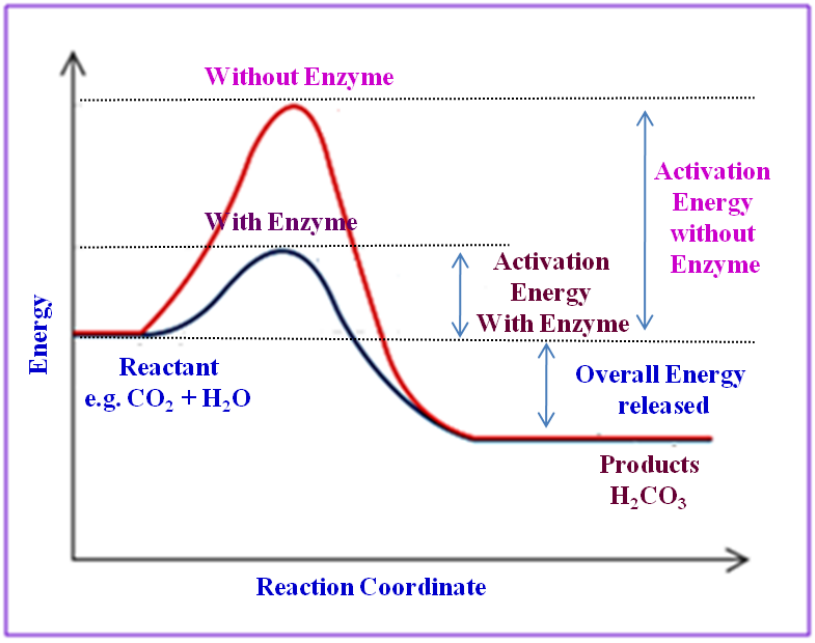


Figure 3.4: Energetic of reaction showing the role of an enzyme in lowering the activation energy.

- **As all catalysts, enzymes also do not change the position of the chemical equilibrium, but only the speed at which it is reached.**

- **Under irreversible reaction conditions the enzyme, in fact, only catalyzes the reaction in the thermodynamically allowed direction.**

3.7. Kinetics of Enezyme Catalysis

- **Definition: The study of the reaction rates and how the rates are affected by changes in experimental conditions of enzyme catalyzed reactions is called *enzyme kinetics*.**

- **Study of enzyme kinetics is an approach to understanding how changes in the physical and chemical environments change function**

- **The rate of a reaction is influenced by several factors:**
  - **concentrations of enzymes, substrates and products**
  - **modifiers like inhibitors, activators or cofactors**
  - **environmental conditions (like temperature and pH)**

- ***Saturation (*Vmax*): It is a*n essential feature of enzyme-catalyzed reactions. It is the stage where an increase in concentrations of substrates the rate increases and approaches a limit where there is no dependence of rate on concentration.**

- **The first scientists to experiment with enzyme kinetics in a more detailed way are Leonor Michaelis and Maud Menten.**

- **Enzyme-Substrate (E-S) Complex: Enzyme binds the transition state structure about 10^{12} times more tightly than it binds the substrate or products. Then, how does an enzyme release product so efficiently?**

  - **As bonds are broken/made at transition state, electronic redistribution can occur → interactions for transition-state stabilization are no longer present → generating a repulsive interaction → Therefore products are poorly bound → lead expulsion of products.**

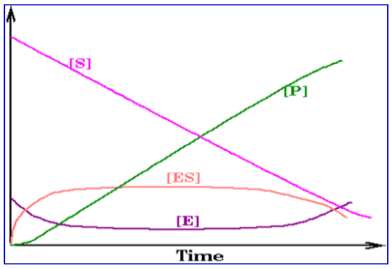
- **Enzymatic reaction:**

Where, E = Enzyme; S = Substrate; [E-S] = Enzyme-Substrate Complex; P = Product; k_1 = Rate of forward reaction; k_{-1} = Rate of reverse reaction; k_2 = Rate of product formation = also known as k_{cat}.

3.7.1. Michaelis-Menten Kinetics

Michaelis-Menten Kinetics

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [E\text{-}S] \xrightarrow{k_2} E + P$$



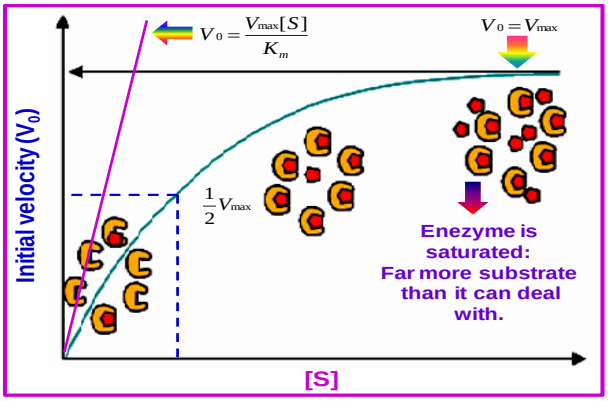
- Rate for product formation: $v = dP/dt = k_2[E\text{-}S]$
- Also, $d[E\text{-}S]/dt = k_1[E][S] - k_{-1}[E\text{-}S] - k_2[E\text{-}S]$
- Conservation of enzyme: $[E] = [E_0] - [E\text{-}S]$
- Steady state assumption: When [S] >> [E] ,

  $[ES] = k_1[E][S]/(k_{-1}+k_2)$
- k_m: A ratio of Rate constants :

  $k_m = k_{-1} + k_2 / k_1$

  k_m = Michaelis-Menten constant
- Initial Velocity (v_o) and [S] :

  The concentration of substrate [S] greatly influences the rate of product formation, i.e. velocity (v) of a reaction. Thus, the effects of [S] on v is complicated by the reversibility of enzyme reactions. To overcome this problem, the use of initial velocity (v_o) measurements are used. At the start of a reaction, [S] is in large excess of [P], thus the initial velocity of the reaction will be dependent on substrate concentration and is measured as the [S] increases until rate becomes constant, i.e.: approaches to a maximum, $V_{max.}$



- **Thus, the Michaelis-Menten Equation is:**

$$V_0 = \frac{V_{max}[S]}{K_m + [S]} = \frac{k_2[E_0][S]}{K_m + [S]}$$

Where, V_0 = Initial velocity; V_{max} = Maximum velocity; [S] = Substrate concentration; K_m = Michaelis-Menten constan $= \frac{k_{-1} + k_2}{k_1}$

3.7.1.1. Significance of Various Parameters in Michaelis-Menten Equation

Significance of Km- Measure of Substrate affinity:

- From Michaelis-Menten equation: If v_o is set equal to 1/2 V_{max}, then the relation $V_{max}/2 = V_{max}[S]/K_m + [S]$ can be simplified to $K_m + [S] = 2[S]$, or $K_m = [S]$. This means that at one half of the maximal velocity, the substrate concentration at this velocity will be equal to the K_m.

- The significance of K_m change depends on the different rate constants and which step is the slowest rate-limiting step. In the simplest assumption, the rate of ES breakdown to product (k_2) is the rate-determining step of the reaction, so $k_{-1} >> k_2$ and $K_m = k_{-1}/k_1$. This relation is also called dissociation constant for the ES complex and can be used as a relative measure of the affinity of a substrate for an enzyme (identical to K_d). However if $k_2 >> k_{-1}$ or k_2 and k_{-1} are similar, then K_m remains more complex and cannot be used as a measure of substrate affinity.

- K_m is a dissociation constant, so the smaller the K_m the stronger the interaction between E and S.

- Each enzyme has a characteristic K_m for a given substrate that show how tight the binding of the substrate is to the enzyme.

- Experimentally, K_m is a useful parameter for characterizing the number and/or types of substrates that a particular enzyme will utilize.

- It is also useful for comparing similar enzymes from different tissues or different organisms. Also, it is the K_m of the rate-limiting enzyme in many of the biochemical metabolic pathways that determines the amount of product and overall regulation of a given pathway.

- Clinically, K_m comparisons are useful for evaluating the effects mutations have on protein function for some inherited genetic diseases.

Significance of V_{max}:

- The values of V_{max} vary widely for different enzymes and can be used as an indicator of an enzymes catalytic efficiency. It does not find much clinical use.

- There are some enzymes that have been shown to have the following reaction sequence:

$$\mathbf{E + S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathbf{[E\text{-}S]} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathbf{[EP]} \overset{k_3}{\rightleftharpoons} \mathbf{E + P}$$

- In this situation, the formation of product is dependent on the breakdown of an enzyme-product complex, and is thus the rate-limiting step defined by k_3.

Definition and Significance of k_{cat}:

- The constant, k_{cat} (sec^{-1}), is also called the turnover number because under saturating substrate conditions, it represents the number of substrate molecules converted to product in a given unit of time on a single enzyme molecule. In practice, k_{cat} values (not V_{max}) are most often used for comparing the catalytic efficiencies of related enzyme classes or among different mutant forms of an enzyme.

- k_{cat}/K_m is the specificity constant - used to rank an enzyme according to how good it is with different substrates.

$$\textbf{If } V_{max} = k_{cat}[E]_{tot}\textbf{, then } V_0 = \frac{k_{cat}[E]_{tot}[S]}{K_m + [S]}$$

- Upper limit for k_{cat}/K_m is rate of diffusion (109 $M^{-1}s^{-1}$)

- Units on k_2 are amount product per amount of enzyme per unit time (also called the “turnover number”). Units on E_0 are amount of enzyme (moles, grams, units) per unit volume; Km has the same units as [S] (mole/liter)].

3.7.1.2. Experimental Rate Parameters of Michaelis-Menten Kinetics

Experimentally Determining Rate Parameters for Michaelis-Menten Kinetics

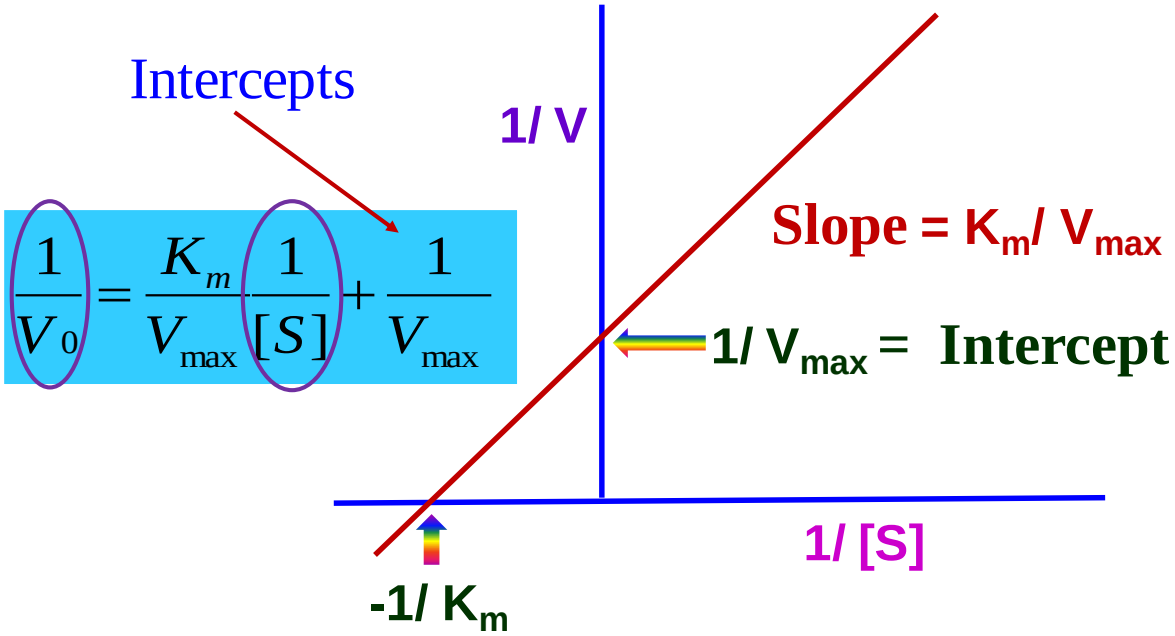
- The Michaelis-Menten Equation is:

$$V_0 = \frac{V_{max}[S]}{K_m\ [S]} = \frac{k_2\ [E_0]\ [S]}{K_m + [S]}$$

- Rearranging the equation into a linear form i.e. if the reciprocal (1/X) of the Michaelis-Menten equation is taken, after algebraic simplification the following linear equation results:

$$\frac{1}{V_0} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}$$

- Plotting $1/V_0$ versus $1/[S]$ will give a straight line as below, the slope of which is K_m/V_{max}, intercept is $1/V_{max}$ and are related to parameter values. This is called Lineweaver-Burk double reciprocal plot.



- **Uses of double reciprocal plot:** The x intercept value is equal to $-1/K_m$. The biggest advantage to using the double reciprocal plot is a more accurate determination of V_{max}, and hence K_m. It is also useful in characterizing the effects of enzyme inhibitors and distinguishing between different enzyme mechanisms.

3.8. Enzyme Inhibition/Inhibitor and Types

Enzyme Inhibition/Inhibitor:

- Blocking an enzyme's activity by chemical molecules which resemble or mimic a particular enzymes substrate(s) is called Enzyme Inhibition and the mole is said to be an Inhibitor. Therefore, many therapeutic drugs are some type of enzyme inhibitor.

- Molecules that bind to enzymes and increase their activity are called *Enzyme Activators.*

- The binding of an inhibitor can stop a substrate from entering the enzyme's active site and/or hinder the enzyme from catalysing its reaction.

- The modes and types of inhibitors have been classified by their kinetic activities and sites of actions.

Types of Inhibition

- **Reversible Inhibition:** reversible inhibitors bind non-covalently (hydrogen bonds, hydrophobic interactions and ionic bonds) and different types of inhibition are produced depending on whether these inhibitors bind the enzyme, the enzyme-substrate complex, or both.

- **Irreversible Inhibition:** Irreversible inhibitors usually react with the enzyme and change it chemically. These inhibitors modify key amino acid residues needed for enzymatic activity.

- Enzyme inhibitors also occur naturally and are involved in the regulation of metabolism.

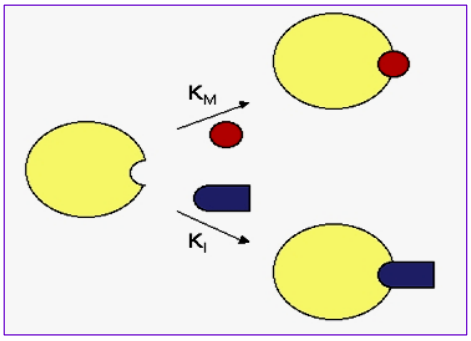
Types of Reversible Inhibition:

(I) Competitive, (II) Non-Competitive, (III) Uncompetitive, and (IV) Mixed Inhibition.

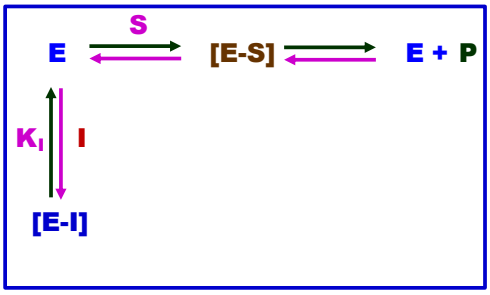
3.8.1. Reversible Enzyme Inhibition

Reversible Enzyme Inhibition

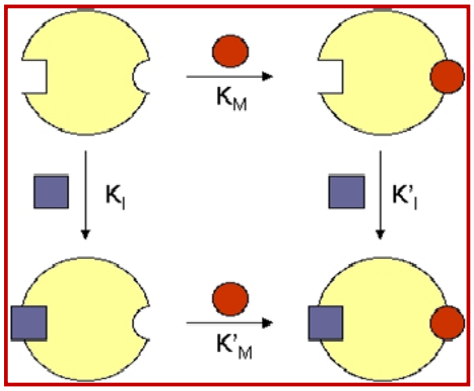
(I) Reversible Competitive Inhibition:



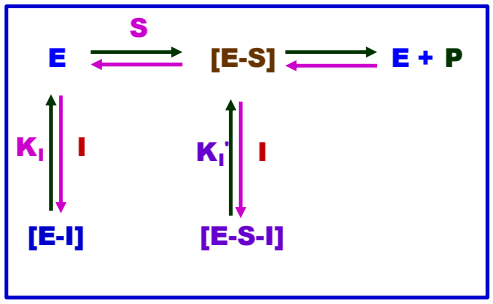
In Competitive Inhibition the substrate and inhibitor cannot bind to the enzyme at the same time. Here, the substrate and inhibitor *compete* for binding to the enzyme's same active site.



(II) Reversible Non-Competitive Inhibition:

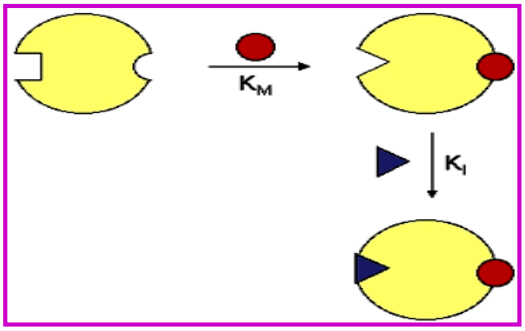


Non-competitive inhibitors cbind both the enzyme (E + I = E-I) and the enzyme-substrate (E-S + I = E-S-I) complex. The inhibitor binds to a site other than the substrate site, and is thus independent of the presence or absence of substrate. This action results in a conformational change in the protein that affects a catalytic step and hence decreases or eliminates enzyme activity (formation of P).

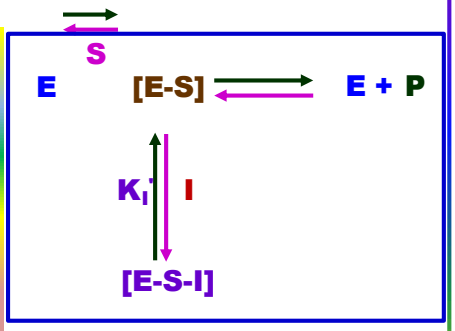


Reversible Enzyme Inhibition

(III) Reversible Uncompetitive Inhibition:

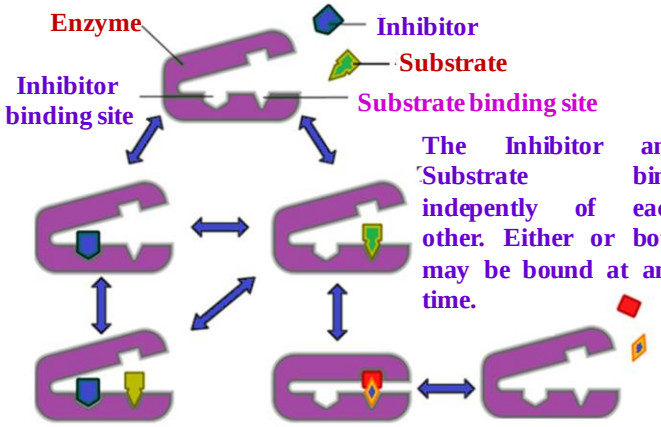


In uncompetitive inhibition the inhibitor can not bind to the free enzyme, but it binds only to the E-S complex. The E-S-I complex thus formed is enzymatically inactive. This type of inhibition is rare, but may occur in multimeric enzyme.



(IV) Reversible Mixed Inhibition:

- **Mixed inhibition** is a combination of two different types of reversible enzyme inhibition – competitive inhibition and uncompetitive inhibition.
- The inhibitor can bind to either the free enzyme or the enzyme-substrate complex ($K_i \neq K_i'$).
- In mixed inhibition, the inhibitor binds to a site different from the active site where the substrate binds. This hamper catalysis in the ES complex (decrease V_{max}).



The Inhibitor and Substrate bind indepently of each other. Either or both may be bound at any time.

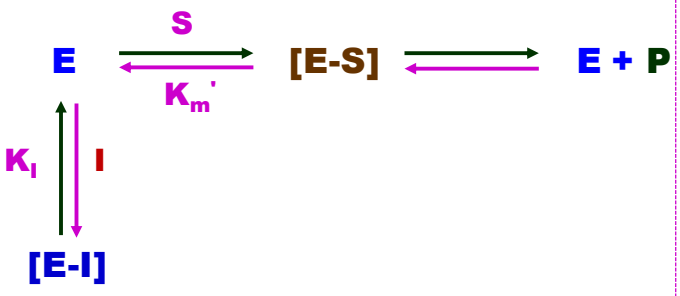
Completion of the reaction, however, depends on enzyme undergoing a conformational change that is blocked by the inhibitor.

3.8.1.1. Kinetics of Reversible Competitive Inhibition

- Competitive Inhibitor is an analog of the substrate, and binds to the active site of the enzyme. In virtually every case, competitive inhibitors bind in the same binding site as the substrate, but same-site binding is not a requirement. A competitive inhibitor could bind to an allosteric site of the free enzyme and prevent substrate binding, as long as it cannot bind to the allosteric site when the substrate is bound.

Kinetics of Reversible Competitive Inhibition

- **Kinetic Equation:**



$$K_m = \frac{[E][S]}{[ES]}$$

$$K_I = \frac{[E][I]}{[EI]}$$

$$[E_0] = [E] + [ES] + [EI]$$

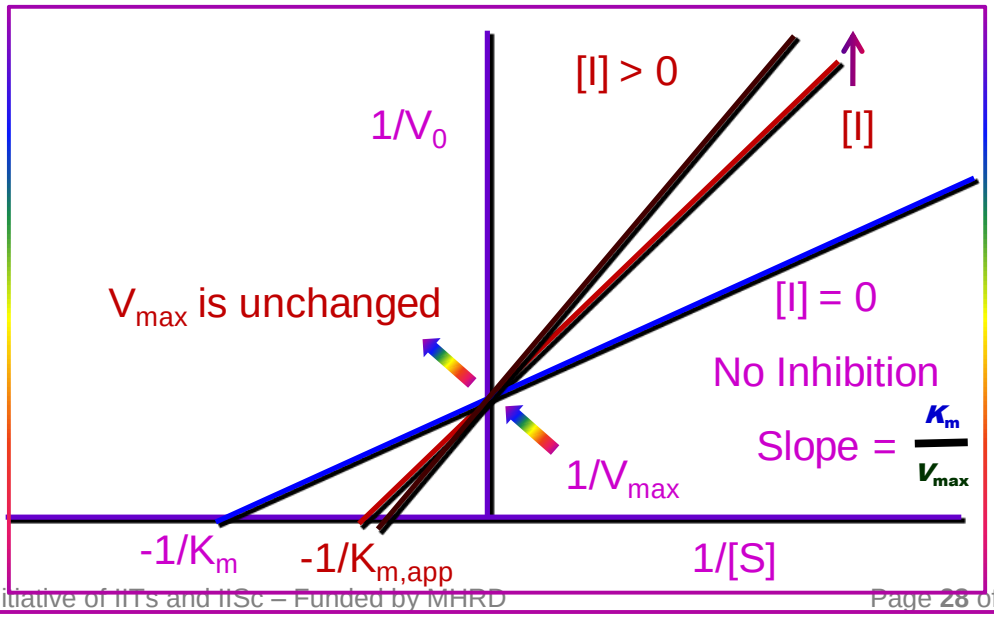
$$V_0 = k_2[ES]$$

- **Rate is given by:**

$$V_0 = \frac{V_{max}[S]}{K_m\left[1 + \frac{[I]}{K_I}\right] + [S]} = \frac{V_{max}[S]}{K_{m,app} + [S]}$$

$$Apparent\ K_m = K_{m,app} = K_m\left[1 + \frac{[I]}{K_I}\right]$$

- What is the magnitude of $K_{m,app}$ relative to K_m and what will be the effect on V_0? How could one run a process to minimize the effects of this type of inhibition?



- Competitive inhibitors compete with the substrate for binding at the active site (as E + I).
- In the double reciprocal plot for a competitive inhibitor acting at the substrate site followings can be noticed: with increasing concentration of inhibitor, the V_{max} does not change; because the presence of the inhibitor can be overcome by higher substrate concentrations.
- However, the K_m of the substrate is increased (the K_d dissociation constant is apparently increased). This is because the concentration of substrate needed to reach Vmax with an inhibitor is greater than the concentration of substrate needed to reach V_{max} without an inhibitor.Increasing value of K_m indicates a direct interaction of the inhibitor in the active site. The change in K_m (Michaelis-Menten constant) is parallel to the alteration in K_d.
- This also reflects the reversible nature of the inhibitor; there is always some concentration of substrate which can displace the inhibitor. Any given competitive inhibitor concentration can be overcome by increasing the substrate concentration in which case the substrate will outcompete the inhibitor in binding to the enzyme.

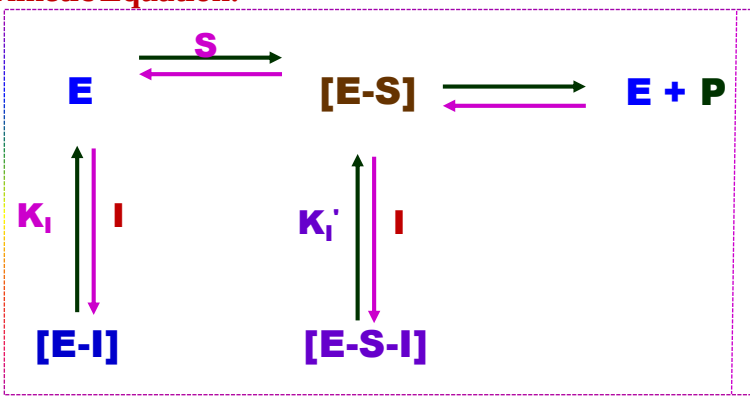
3.8.1.2. Kinetics of Reversible Non-Competitive Inhibition

- Non-competitive inhibitors combine with both the enzyme (E + I) and the enzyme-substrate (ES + I) complex. The inhibitor binds to a site other than the substrate site, and is thus independent of the presence or absence of substrate. This action results in a conformational change in the protein that affects a catalytic step and hence decreases or eliminates enzyme activity, i.e. formation of P.
- In the reciprocal plot, it is clear that a non-competitive inhibitor does not affect the binding of the substrate (K_m), but results in a decrease in V_{max}. This can be explained by the fact that since inhibitor bound to an enzyme inactivates it, the more [EI] formed will lower [ES] and thus lower the overall rate of the reaction V_{max}.
- In the presence of a non-competitive inhibitor, the apparent enzyme affinity is equivalent to the actual affinity. In terms of Michaelis-Menten kinetics, $K_m^{app} = K_m$. This can be seen as a consequence of Le Chatelier's Principle because the inhibitor binds to both the enzyme and the enzyme-substrate complex equally so that the equilibrium is maintained. However, since some enzyme is always inhibited from converting the substrate to product, the effective enzyme concentration is lowered.

- When both the substrate and the inhibitor are bound, the enzyme-substrate-inhibitor complex cannot form product and can only be converted back to the enzyme-substrate complex or the enzyme-inhibitor complex. Non-competitive inhibition is distinguished from general mixed inhibition in that the inhibitor has an equal affinity for the enzyme and the enzyme-substrate complex.
- This type of inhibition reduces the maximum rate of a chemical reaction without changing the apparent binding affinity of the catalyst for the substrate (K_m^{app}). 6-hydroxyflavone is an example of Non-competitive inhibitors of CYP2C9 enzyme which bind in the allosteric binding site.

Kinetics of Reversible Non-Competitive Inhibition

- **Kinetic Equation:**



$$K_m = \frac{[E][S]}{[ES]} = \frac{[EI][S]}{[ESI]}$$

$$K_I = \frac{[E][I]}{[EI]} = \frac{[ES][I]}{[ESI]}$$

$$[E_0] = [E] + [ES] + [EI] + [ESI]$$

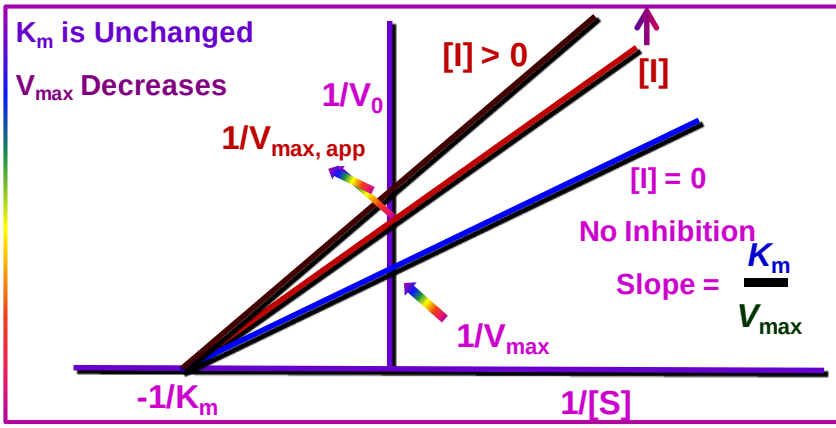
$$V_0 = k_2[ES]$$

- **Rate is given by:**

$$V_0 = \frac{V_{max}}{\left[1 + \frac{[I]}{K_I}\right]\left[1 + \frac{K_m}{[S]}\right]} = \frac{V_{max,app}}{\left[1 + \frac{K_m}{[S]}\right]}$$

$$Apparent\ V_{max} = V_{max,app} = \frac{V_{max}}{\left[1 + \frac{[I]}{K_I}\right]}$$

- **Plot of $1/V_{max}$ vs. $1/[S]$**



$$V_0 = k_{cat,\,app}[E_0]\frac{[S]}{[K_m + [S]]}$$

$$k_{cat,\,app} = k_{cat}\frac{K_I}{[K_I + [I]]}$$

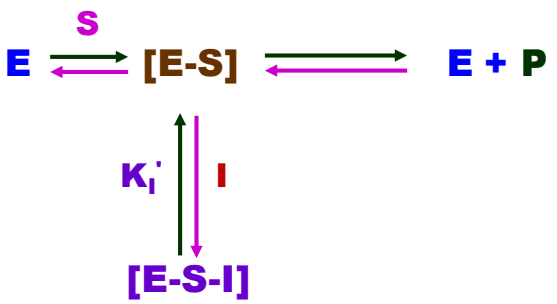
3.8.1.3. Kinetics of Reversible Un-Competitive Inhibition

- **Uncompetitive inhibition**, (**anti-competitive inhibition**), takes place when an enzyme inhibitor binds only to the complex formed between the enzyme and the substrate (the E-S complex).

Kinetics of Reversible UnCompetitive Inhibition

- **Uncompetitive inhibition**, (**anti-competitive inhibition**), takes place when an enzyme inhibitor binds only to the complex formed between the enzyme and the substrate (the E-S complex).

- **Kinetic Equation:**



$$K_m = \frac{[E][S]}{[ES]}$$

$$K_I = \frac{[E][I]}{[EI]}$$

$$[E_0] = [E] + [ES] + [ESI]$$

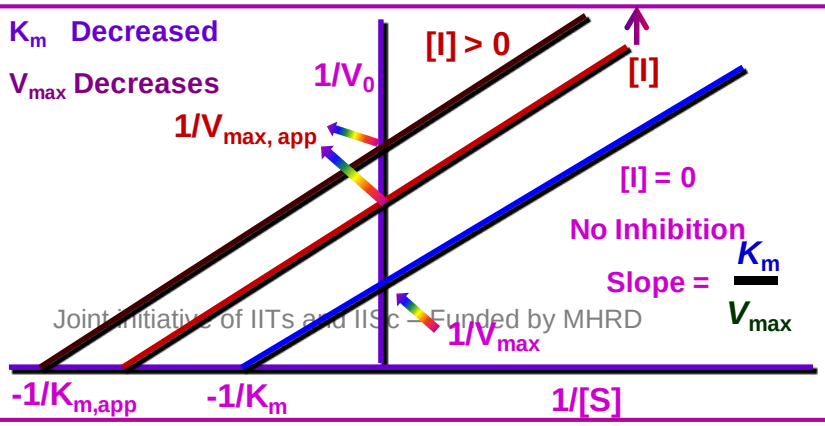
$$V_0 = k_2[ES]$$

- **Rate is given by:**

$$V_0 = \frac{\frac{V_{max}}{\left(1+\frac{[I]}{K_I}\right)}[S]}{\frac{K_m}{\left[1+\frac{[I]}{K_I}\right]}+[S]} = \frac{V_{max\,app}[S]}{K_{m,app}+[S]}$$

$$\frac{1}{V_0} = \frac{K_m}{[V_{max}[S]]} + \frac{1+\frac{[I]}{K_I}}{V_{max}}$$

- **In the reciprocal plot:**
  - The Lineweaver–Burk equation for an uncompetitive inhibitor produces a line parallel to the original enzyme-substrate plot, but with a higher y-intercept (due to the inhibition term).
  - Decreasing the $K_{m,app}$ → enzyme affinity of E and S has increased.



$$Apparent\ V_{max} = V_{max,app} = \frac{V_{max}}{\left[1+\frac{[I]}{K_I}\right]}$$

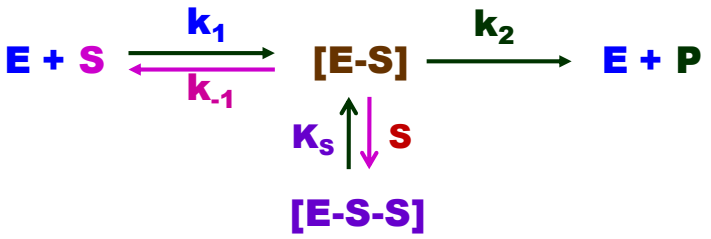
$$Apparent\ K_m = K_{m,app} = \frac{K_m}{\left[1+\frac{[I]}{K_I}\right]}$$

3.8.1.3.1 Substrate Inhibition: A Special Case of Uncompetitive Inhibition

- A special case of uncompetitive inhibition is **substrate inhibition** which occurs at high substrate concentrations in about 20% of all known enzymes (e.g. invertase is inhibited by sucrose). It is primarily caused by more than one substrate molecule binding to an active site meant for just one, often by different parts of the substrate molecules binding to different subsites within the substrate binding site. If the resultant complex is inactive this type of inhibition causes a reduction in the rate of reaction, at high substrate concentrations.

Substrate Inhibition: A Special Case of Uncompetitive Inhibition

- **Kinetic Equation:**

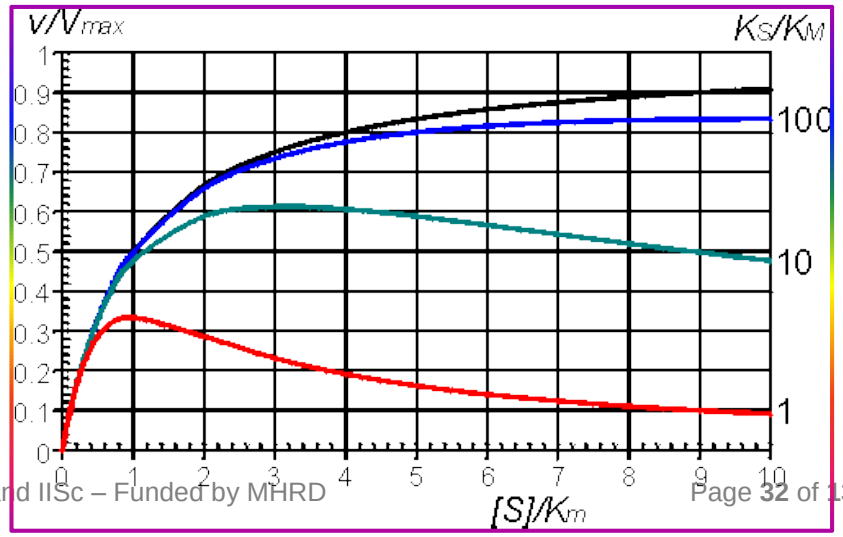


$$K_S = \frac{[ES][S]}{[ESS]}$$

$$V_0 = \frac{V_{max}[S]}{K_m + [S]\left[1 + \frac{[S]}{K_S}\right]}$$

- The assumption is made that ESS may not react to form product. Even quite high values for K_S lead to a levelling off of the rate of reaction at high substrate concentrations, and lower K_S values cause substantial inhibition.

- The effect of substrate inhibition on the rate of an enzyme-catalysed reaction is shown below. A comparison is made between the inhibition caused by increasing K_S relative to K_m. **(__) No inhibition, K_S/K_m >> 100**; **(__) Inhibition, K_S/K_m = 100**; **(__) Inhibition, K_S/K_m = 10**; **(__) Inhibition, K_S/K_m = 1**. By the nature of the binding causing this inhibition, it is unlikely that $K_S/K_m < 1$.



3.8.1.4. Meaning of K_I

Meaning of K_I

Meaning of K_i:

- For reversible inhibitors, a term K_I can be determined.
- For competitive inhibitors, the following relation can be used: $K_{m+I} = K_m (1 + [I] / K_I)$; (where K_{m+I} is the determined K_m in the presence of [I]).
- Determining the K_I for other inhibitor types is related but much more complex.

Uses of K_I:

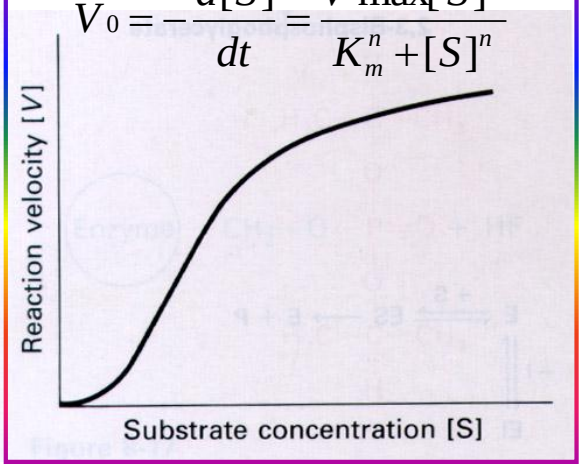
- **K_I values** are used to characterize and compare the effectiveness of inhibitors relative to K_m. This parameter is especially useful and important in evaluating the potential therapeutic value of inhibitors (drugs) of a given enzyme reaction. For example, K_I values are used for comparison of the different types of HIV protease inhibitors. **In general, the lower the K_I value, the tighter the binding, and hence the more effective an inhibitor is.**

3.8.1.5. Allosteric Enzyme Kinetics

Allosteric Enzyme Kinetics

- In an enzyme with more than one substrate binding site, binding of one substrate molecule affects the binding of another.
- n>1, cooperation; n<1, interference.
- Shape of rate curve is sigmoidal.

$$V_0 = -\frac{d[S]}{dt} = \frac{V\max[S]^n}{K_m^n + [S]^n}$$



3.8.2. Irreversible Inhibition

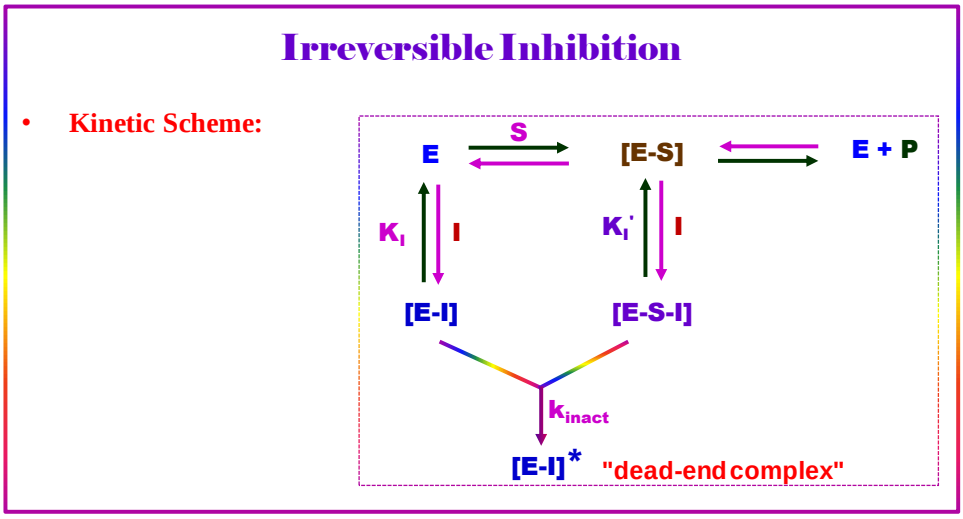
- Irreversible inhibitors are bind via covalent linking to the enzyme causing modification of the enzyme and inactivating it.

- Many Enzymes contain -SH, -OH,- or -COOH groups as part of their active sites, any chemical which can react with them acts as an irreversible inhibitor. Heavy metals such as Ag^{+}, Hg^{2+}, Pb^{2+} have strong affinities for -SH groups.

- Irreversible inhibitors display time-dependent inhibition and their potency therefore cannot be characterised by an IC_{50} value, because the amount of active enzyme at a given concentration of irreversible inhibitor will be different depending on the time of pre-incubation.

- By plotting the log of % activity vs. time, $k_{obs}/[I]$ parameter can be derived, where k_{obs} is the observed pseudo-first order rate of inactivation and $[I]$ is the concentration of inhibitor.

- The $k_{obs}/[I]$ parameter is valid as long as the inhibitor does not saturate binding with the enzyme wherein $k_{obs} = k_{inact}$.



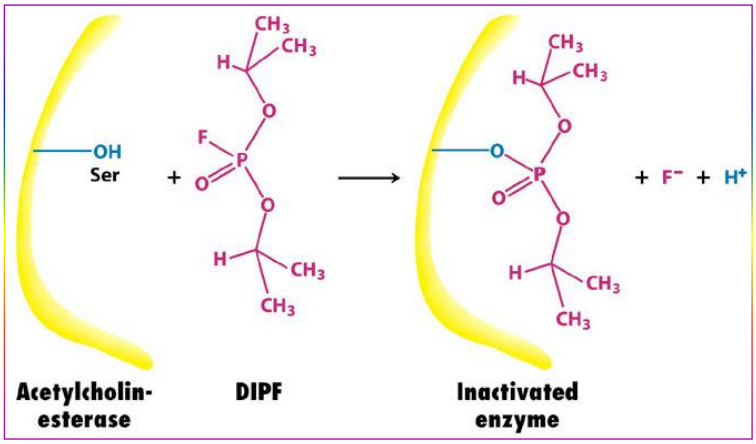
- **Types of Irreversible Inhibition:**
  - Group-specific covalent modifying agents
  - Affinity labels
  - Transition state analogs
  - Suicide inhibitors (mechanism-based inhibitors.

3.8.2.1. Examples of Irreversible Inhibitions

Examples of Different Types of Irreversible Inhibition

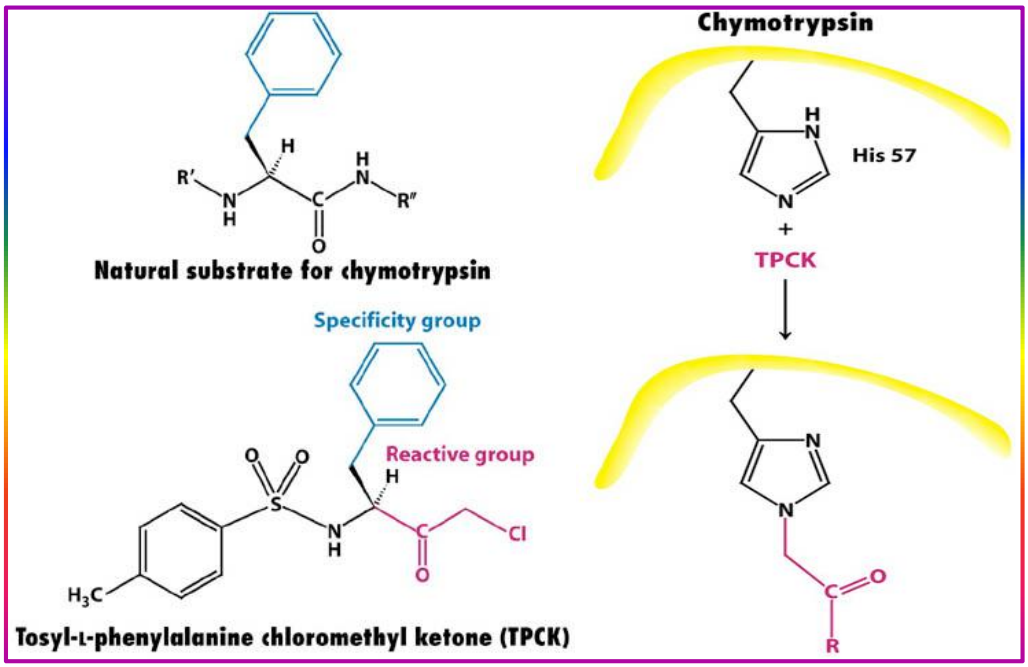
1. Group specific covalent modifying agents:

- This type of inhibitors react with specific type of enzyme functional group (e.g., Ser-OH, or Cys-SH, or His imidazole) on any enzyme/protein.
- **Example: Diisopropylphosphofluoridate (DIPF),** potent nerve gas (poison) which reacts with specific, reactive Ser-OH on many enzymes such as catalytic -OH group of acetylcholinesterase at synaptic junctions → modified enzyme inactivated.



2. Affinity Labels:

- structural similarity to substrate "guides" reagent to active site
- reaction at active site covalently inactivates enzyme
- **Example: Tosyl phenylalanyl chloromethylketone (TPCK)**--phenyl group binds in substrate specificity site of chymotrypsin

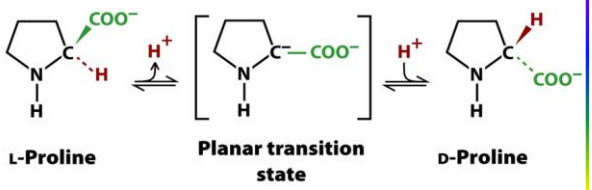


Examples of Different Types of Irreversible Inhibition

3. Transition State Analogs:

- structurally similar to transition state, which binds even more tightly to enzyme than substrate binds, so very high affinity for active site
- **Transition state analogs useful for:**
  - understanding catalytic mechanisms (clues about structure of transition state).
  - very specific inhibitors of enzymes (pharmaceutical applications)
  - antigens for immunizing lab animals to generate antibodies with binding sites complementary to the transition state such that the antibodies themselves have catalytic activity ("abzymes")

Proline Racemase (planar transition state)

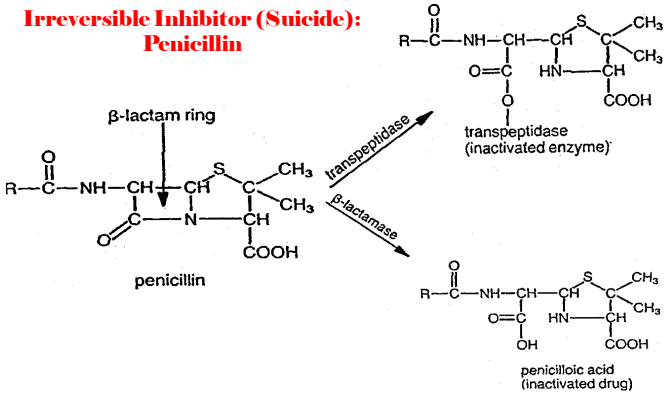


Transition State Analogs of Proline

4. Suicide Inhibition-Mechanism-Based Inhibition:

- Structural similarity to substrate "guides" reagent to active site.
- Enzyme *treats it as a substrate, starting chemical catalytic process* with inhibitor.
- *Chemical mechanism itself leads enzyme to react covalently with* inhibitor, thus "committing suicide".
- Mechanism-based inhibition depends on *chemical mechanism of catalysis.*
- **Example:** penicillin--inhibits an enzyme, a transpeptidase, required for bacterial cell wall synthesis.

Irreversible Inhibitor (Suicide): Penicillin



Activity of Penicillin (an antibiotic):

- both a transition-state analog and a suicide substrate
- covalently inhibits a transpeptidase (enzyme) involved in bacterial cell wall synthesis (eukaryotic cells don't have this enzyme)
- Normal transpeptidase catalytic mechanism: nucleophilic attack of enzyme Ser–OH on substrate, making a covalent acyl-enzyme intermediate
- Covalent intermediate continues in enzyme-catalyzed reaction to form peptide cross-link in peptidoglycan structure of cell wall, regenerating free enzyme for another round of catalysis.
- Penicillin resembles transition state in structure, so penicillin a) binds very tightly and b) is very reactive.
- Normal catalytic mechanism makes covalent intermediate with penicillin → enzyme is inactivated because of its own catalytic activity – it committed suicide!

3.9. The Catalytic Triad

Proteases, commonly called peptidases, represent approximately 2% of the total number of proteins present in all types of organisms. There are about 500 human genes that encode peptidases and their homolog. Many of these enzymes are of medical importance, and are potential drug targets that originate from the human genome or from the genome of the disease-causing organism.

Classification of Proteases: The homologous peptidases are divided into clans and families. Families are grouped in a clan according to tertiary structure comparisons. Clans arise from a common ancestor. Four distinct mechanisms are known that linked to the serine, cysteine, aspartic and metallopeptidases, respectively. The most conspicuous difference in the mechanisms is the presence or absence of a covalent acyl-enzyme intermediate on the reaction pathway.

Catalysis by Proteases: The catalyses of serine and cysteine peptidases involve the covalent intermediate (ester and thiolester, respectively). During hydrolysis carried out by aspartic and the metallopeptidase catalyses, the substrate is attacked directly by a water molecule rather than by a serine or cysteine residue. In spite of the differences, the basic catalytic features of the different clans are common. Hydrolysis of the peptide bond is an addition-elimination reaction involving a tetrahedral intermediate. All three heavy atoms of the peptide bond are directly implicated in the catalytic reaction by interacting with appropriate enzymatic groups.

3.9.1. The Catalytic Mechanism: The Catalytic Triad:

A **catalytic triad** refers to the three amino acid residues found inside the active site of certain protease enzymes: serine (S), aspartate (D), and histidine (H). They work together to break peptide bonds on polypeptides. In general terms, *catalytic triad* can refer to any set of three residues that function together and are directly involved in catalysis. Because enzymes fold into complex three-dimensional shapes, the residues of a catalytic triad are brought close together in the tertiary structure.

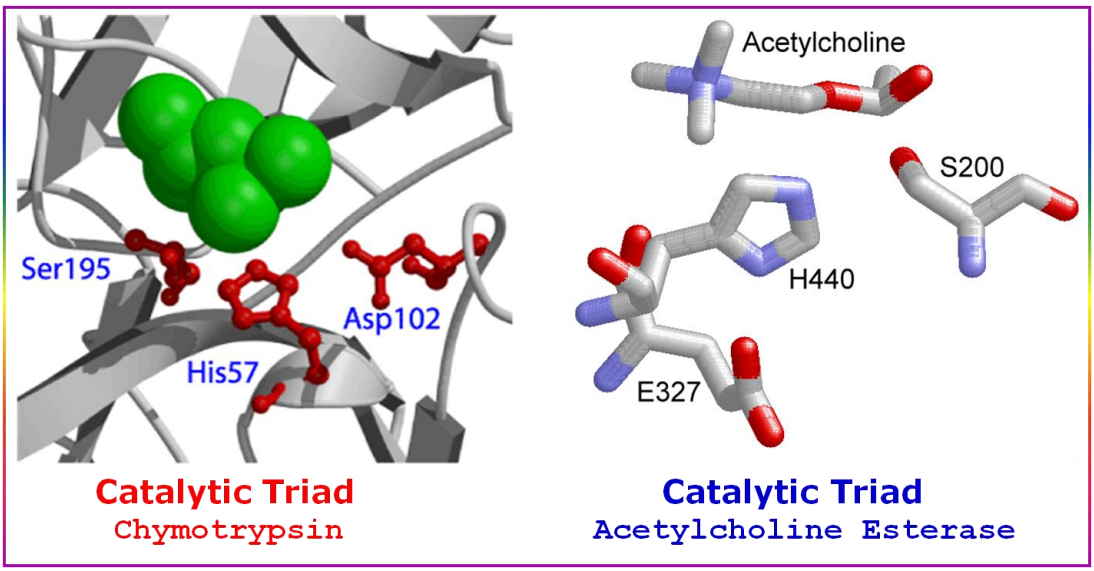


Figure 3.5: The Chymotrypsin and Acetylcholine Esterase Catalytic Triads.

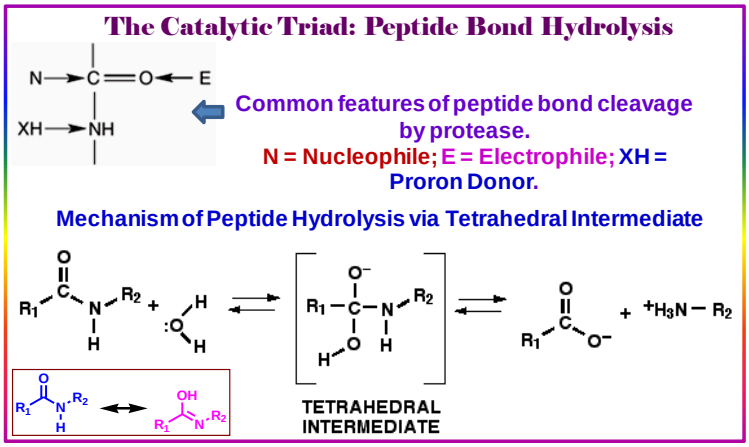
Serine proteases are a class of proteolytic enzymes whose catalytic mechanism is based on an active-site serine residue. Serine proteases are one of the best-characterized families of enzymes. This family includes *trypsin, chymotrypsin, elastase,* (Digestive enzyme) *thrombin (*crucial enzyme in the blood-clotting cascade*), subtilisin (*bacterial protease*), plasmin(*breaks down the fibrin polymers of blood clots*), tissue plasminogen activator (*cleaves the proenzyme *plasminogen*, yielding plasmin*),* and other related enzymes. Finally, although not itself a protease, *acetylcholinesterase is a serine esterase* and is related mechanistically to the serine proteases. It degrades the neurotransmitter acetylcholine in the synaptic cleft between neurons.

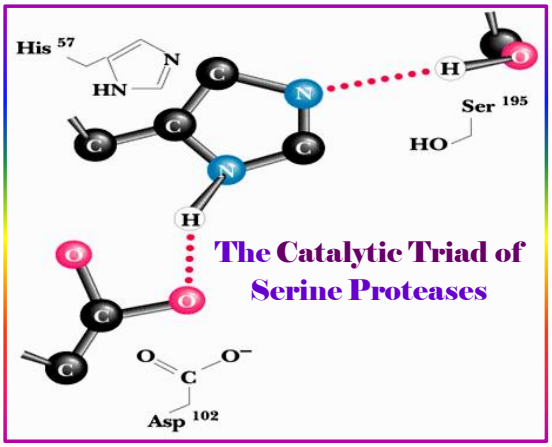
3.9.2. Trypsin, Chymotrypsin, and Elastase: The Digestive Serine Proteases

Trypsin, chymotrypsin, and elastase all carry out the same reaction — the cleavage of a peptide chain — and although their structures and mechanisms are quite similar, they display very different specificities. Trypsin cleaves peptides on the carbonyl side of the basic amino acids, arginine or lysine. Chymotrypsin prefers to cleave on the carbonyl side of aromatic residues, such as phenylalanine and tyrosine. Elastase is not as specific as the other two; it mainly cleaves peptides on the carbonyl side of small, neutral residues. These three enzymes all have similar sequences and three-dimensional structures. In Chymotrypsin, Serine195 binds to the substrate polypeptide to the side of a phenylalanine, tryptophan, or tyrosine residue closer to the C-terminus, holding it in place. Aspartate (D102) and Histidine (H57) then hydrolyze the bond.

Catalytic Triad
The Serine Proteases
(Trypsin, chymotrypsin, elastase, thrombin, subtilisin, plasmin, TPA)

- Proteases catalyze hydrolysis of peptide bonds.
- Equilibrium lies far to the right, but in the absence of a catalyst the reaction is extremely slow.
- Peptide bonds are thus "kinetically stable", or metastable.
- Mechanism of uncatalyzed reaction is simple nucleophilic attack by the H_2O on the carbonyl carbon of the peptide bond, forming a tetrahedral intermediate which then breaks down as the amine "half" of the original peptide leaves.
- Partial double bond character of peptide bond makes its carbonyl carbon poor reactive in uncatalyzed reaction → rate is slow.

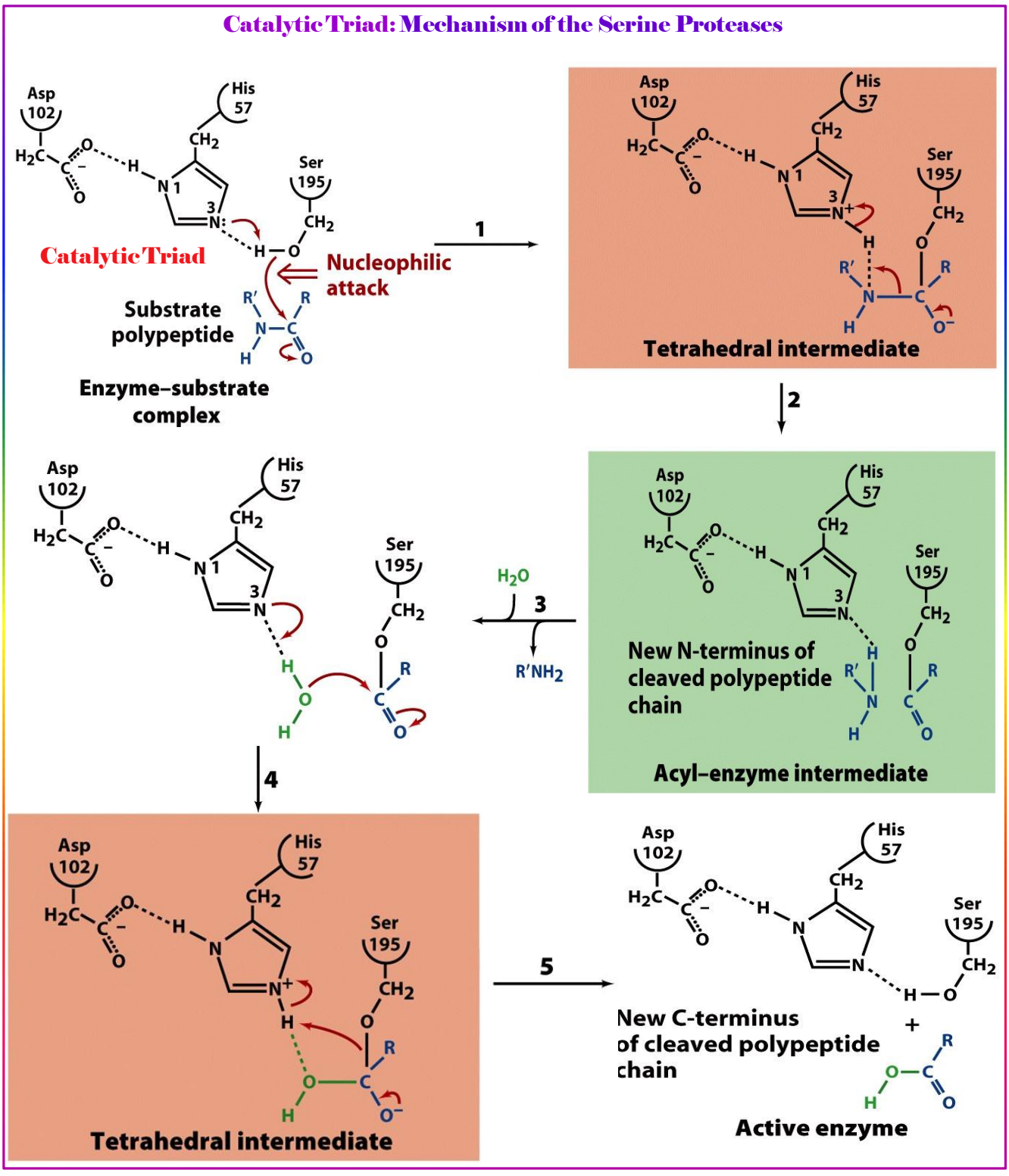


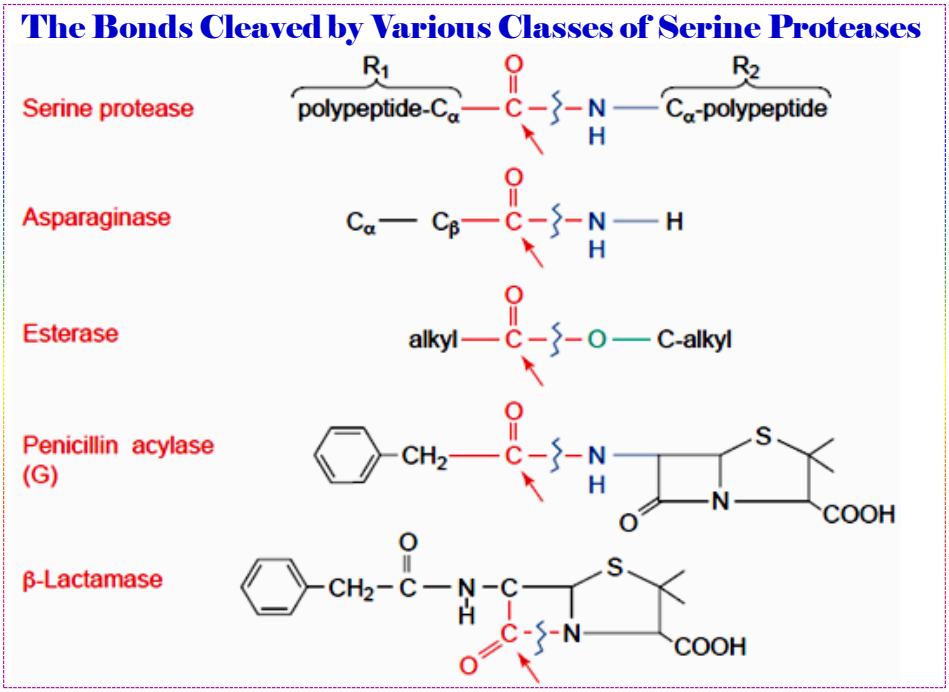


- Catalytic task of proteases is to make that normally unreactive carbonyl group more susceptible to nucleophilic attack by H_2O.
- Serine proteases are *endo*proteases. The serine protease mechanism illustrates not only proximity and orientation, but also: (a) Transition state stabilization, (b) Covalent catalysis, involving a catalytic triad of Asp, His and Ser in the active site, (c) general acid-base catalysis, and (d) electrostatic catalysis.
- Chymotrypsin enhances rate of peptide bond hydrolysis by a factor of at least 10^9
- All serine proteases have in the active site 3 amino acid residues known as the catalytic triad.
- All involve a serine in catalysis - thus the name. Ser is part of a "catalytic triad" of Ser, His, Asp
- Serine proteases are homologous, but locations of the three crucial residues differ somewhat
- Three residues are numberd always as His-57, Asp-102, Ser-195 .
- **The catalytic triad, an oxyanion hole, a specificity pocket and a nonspecific binding region, are all common features of the active sites of these enzymes.**

Serine Protease Mechanism
(A mixture of covalent and general acid-base catalysis)

- Asp-102 functions only to orient His-57
- His-57 acts as a general acid and base
- Ser-195 forms a covalent bond with peptide to be cleaved
- Covalent bond formation turns a trigonal C into a tetrahedral C
- The tetrahedral oxyanion intermediate is stabilized by N-Hs of Gly-193 and Ser-195.


Catalytic Triad: Mechanism of the Serine Proteases
Asp 102
His 57
Ser 195
Catalytic Triad
Nucleophilic attack
Substrate polypeptide
Enzyme–substrate complex
1
Tetrahedral intermediate
2
New N-terminus of cleaved polypeptide chain
Acyl–enzyme intermediate
3
H_2O
$R'NH_2$
4
Tetrahedral intermediate
5
New C-terminus of cleaved polypeptide chain
Active enzyme



3.10. Enzyme Inhibiton and Drug design

Introduction: The drug is most commonly a small organic molecule that generates biochemical and physiological effect on patient's body when it enters. Drug does not impart any new function on any system, cell or organ. It only alters the pace of ongoing cellular activity must by interacting with the biomolecules such as a protein present in cell which in turn results in a therapeutic effect to the patient. To generate a specific response drug must interact specifically with a specific biomolecules i.e. drug must interact with a specific target to show its potency. Therefore, to have a potent drug candidate for a specific disease, one must have to design a drug rationally. Now, the cause of disease is the

malfunctioning of certain biomolecules like, gene, protein or enzymes or because of foreign organism (microbes). Finding the diseases related target, one can administer drug. Thus, Drug design, (rational drug design or structure based drug design), is the innovative process of finding new medications based on the knowledge of the biological target. Thus, in the most basic sense, drug design involves design of small molecules that are complementary in shape and charge to the biomolecular target to which they interact and therefore will bind to it. That a drug can only show its effect when it interacts with its target can be proved by the example of knockout mice. A lack of effect of a drug in mice lacking a particular target can provide strong support that the effects of the drug are mediated by that target.

3.10.1. What Is Drug Targets?

Most drugs work because by binding to the target in the cell, they can either block the physiological function of the target, or mimics its effect. If a drug causes the target to respond in the same way as the naturally occurring substance, then the drug is referred to as an agonist. Antagonists are drugs that interact selectively with the target but do not lead to an observed effect. Instead they reduce the action of an agonist at the target site involved. So what is a drug target?

A “target” of a drug may be defined as a biomolecular structure that undergoes a specific interaction with chemicals (drugs) because they are administered to treat or diagnose a disease. The interaction has a connection with the clinical effect(s). Almost all the biomolecules acting as a drug target have a specific three-dimensional structure which allows only substances that fit precisely to attach to it. According to this definition we are restricting the target as a static bio-molecular architecture. However, Life process, including disease state is a dynamic because we do not yet directly observe the interactions of drugs and targets and only what we notice is the biochemical response they produce.

For most drugs, many targets were identified. Consequently, for drug administration we must have to rely on the existing data that showed some connection between the interaction of the drug with the biochemical structure of the target and the beneficial clinical effect(s). A chemical with a certain reactivity or binding property is used as a drug because of its clinical effects. However it would be challenging to prove that a certain molecular interaction triggers the drug effect(s). That a drug can only show its effect when it interacts with its target can be proved by the example of knockout mice. A lack of effect of a drug in mice lacking a particular target can provide strong support that the effects of the drug are mediated by that target. All drugs somehow interfere with signal transduction, receptor signaling and biochemical equilibrium. Also several drugs interact with more than one target. So, there will be simultaneous changes in several biochemical signals, and there will be feedback reactions of the pathways disturbed, drug action is a dynamic process. All the discussion about the target

led to conclude that a clinically relevant 'target' might consist not of a single biochemical entity, but the simultaneous interference of a number of pathways, enzymes and so on. Only this will give a net clinical effect that might be considered beneficial.

Greater knowledge of how drugs interact with the body (mechanisms of action, drug–target interactions) has led to a reduction of established drug doses and inspired the development of newer, highly specific drug substances with a known mechanism of action. Considering system biology, dynamic nature of drug target and drug response we should say at molecular level that the nature of target is in-between “macro”- and “micro”-target. Therefore a drug target can best be defined as a key bio-moleculear entity of a cell involved in a particular metabolic or signaling pathway that is specific to a disease condition or pathology, or to the infectivity or survival of a microbial pathogen.

3.10.2. Types of Drug Targets

The completion of the human genome project has set the stage to the biomedical researchers to revolutionize the medical advancement for disease care. In 1997, 100,000 protein-coding sequences were hypothesized to exist in the human genome. Many of these were supposed to hold the keys to treat disease. However, the number of molecular targets 'hit' by all marketed drug substances were estimated by Drews and Ryser as about 482. About 8,000 targets of pharmacological interest were estimated in 2002, after the sequencing of the human genome. Out of that nearly 5,000 target were found as potentially hit by traditional drug substances, about 2,400 by antibodies and about 800 by protein pharmaceuticals. On the basis of ligand-binding studies, 399 molecular targets were identified belonging to 130 protein families, and 3,000 targets for small-molecule drugs in the human genome.

Despite the existence of enormous number of drug targets, drug discovery generally focuses on following first seven major categories of drug targets. However, there are some drug in present market which shows various physicochemical mechanisms of action on target and some drugs with biological response but their target or the mechanism of action is yet to be invented fully.

1. **Enzymes (Table 1)**
2. **Substrates, metabolites and proteins**
3. **Receptors/Nuclear Hormone Receptors**
4. **Ion channels**
5. **Transport proteins**
6. **DNA/RNA and the ribosome**
7. **Targets of monoclonal antibodies**
8. **Targets of Various physicochemical mechanisms**
9. **Unknown Targets of some drugs**

Drugs/Targets with Unknown Mechanism

4-Aminosalicylic acid | Alendronate | Ambroxol | Arsenic trioxide | Becaplermin | Bexarotene | Chloral hydrate | Clofazimine | Dactinomycin (RNA synthesis inhibitor) | Dapsone (folic acid synthesis inhibitor) | Diethyl carbamazine | Diethyl ether | Diloxanide | Dinitric oxide | Ethambutol | Gentian violet | Ginkgolides | Griseofulvin | Halofantrine | Halothane | Hydrazinophthalazine | Limefantrine (antimalarial; prevents haem polymerization) | Levetiracetam | Mebendazole | Methyl-(5-amino-4-oxopentanoate) | Niclosamide | Pentamidine | Podophyllotoxin | Procarbazine | Selenium sulphide

Some approaches of drug action attempt to inhibit the functioning of the pathway in the diseased state by causing a key molecule/bio-molecule to stop functioning. Thus, drugs may be so design as bind to the active region and inhibit this key molecule. Another approach of drug action enhances the normal pathway by promoting specific molecules in the normal pathways that may have been affected in the diseased state. In addition, these drugs should also be designed in such a way as not to affect any other important off-target molecules or anti-targets that may be similar in appearance to the target molecule. Because, drug interactions with off-target molecules may leads to undesirable side effects causing risk.

Thus, it is clear that in drug discovery, target classes influence everything from automation engineering to the design of chemical libraries. Here, we will discuss about one target class, enzymes, and provide an assessment of what kinds of enzyme inhibitors are potent drug candidate.

Table 1: List of Enzymes as Drug/Inhibitor Targets

Type	Activity of drug	Drug examples
Oxidoreductases		
Aldehyde dehydrogenase	Inhibitor	Disulfiram
Monoamine oxidases (MAOs)	MAO-A inhibitor	Tranylcypromine, moclobemide
	MAO-B inhibitor	Tranylcypromine
Cyclooxygenases (COXs)	COX1 inhibitor	Acetylsalicylic acid, profens, acetaminophen and dipyrone (as arachidonylamides)

Type	Activity of drug	Drug examples
	COX2 inhibitor	Acetylsalicylic acid, profens, acetaminophen and dipyrone (as arachidonylamides)
Vitamin K epoxide reductase	Inhibitor	Warfarin, phenprocoumon
Aromatase	Inhibitor	Exemestane
Lanosterol demethylase (fungal)	Inhibitor	Azole antifungals
Lipoxygenases	Inhibitor	Mesalazine
	5-lipoxygenase inhibitor	Zileuton
Thyroidal peroxidase	Inhibitor	Thiouracils
Iodothyronine-5' deiodinase	Inhibitor	Propylthiouracil
Inosine monophosphate dehydrogenase	Inhibitor	Mycophenolate mofetil
HMG-CoA reductase	Inhibitor	Statins
5α-Testosterone reductase	Inhibitor	Finasteride, dutasteride
Dihydrofolate reductase (bacterial)	Inhibitor	Trimethoprim
Dihydrofolate reductase (human)	Inhibitor	Methotrexate, pemetrexed
Dihydrofolate reductase (parasitic)	Inhibitor	Proguanil
Dihydroorotate reductase	Inhibitor	Leflunomide
Enoyl reductase (mycobacterial)	Inhibitor	Isoniazid
Squalene epoxidase (fungal)	Inhibitor	Terbinafin
Δ14 reductase (fungal)	Inhibitor	Amorolfin
Xanthine oxidase	Inhibitor	Allopurinol
4-Hydroxyphenylpyruvate dioxygenase	Inhibitor	Nitisinone
Ribonucleoside diphosphate reductase	Inhibitor	Hydroxycarbamide
Transferases		
Protein kinase C	Inhibitor	Miltefosine
Bacterial peptidyl transferase	Inhibitor	Chloramphenicol

Type	Activity of drug	Drug examples
Catecholamine-*O*-methyltransferase	Inhibitor	Entacapone
RNA polymerase (bacterial)	Inhibitor	Ansamycins
Reverse transcriptases (viral)	Competitive inhibitors	Zidovudine
	Allosteric inhibitors	Efavirenz
DNA polymerases	Inhibitor	Acyclovir, suramin
GABA transaminase	Inhibitor	Valproic acid, vigabatrin
Tyrosine kinases	PDGFR/ABL/KIT inhibitor	Imatinib
	EGFR inhibitor	Erlotinib
	VEGFR2/PDGFRβ /KIT/FLT3	Sunitinib
	VEGFR2/PDGFRβ /RAF	Sorafenib
Glycinamide ribonucleotide formyl transferase	Inhibitor	Pemetrexed
Phosphoenolpyruvate transferase (MurA, bacterial)	Inhibitor	Fosfomycin
Human cytosolic branched-chain aminotransferase (hBCATc)	Inhibitor	Gabapentin
Hydrolases (proteases)		
Aspartyl proteases (viral)	HIV protease inhibitor	Saquinavir, indinavir
Hydrolases (serine proteases)		
Unspecific	Unspecific inhibitors	Aprotinine
Bacterial serine protease	Direct inhibitor	β-lactams
Bacterial serine protease	Indirect inhibitor	Glycopeptides
Bacterial lactamases	Direct inhibitor	Sulbactam
Human antithrombin	Activator	Heparins
Human plasminogen	Activator	Streptokinase
Human coagulation factor	Activator	Factor IX complex, Factor VIII
Human factor Xa	Inhibitor	Fondaparinux
Hydrolases (metalloproteases)		
Human ACE	Inhibitor	Captopril

Type	Activity of drug	Drug examples
Human HRD	Inhibitor	Cilastatin
Human carboxypeptidase A (Zn)	Inhibitor	Penicillamine
Human enkephalinase	Inhibitor	Racecadotril
Hydrolases (other)		
26S proteasome	Inhibitor	Bortezomib
Esterases	AChE inhibitor	Physostigmine
	AChE reactivators	Obidoxime
	PDE inhibitor	Caffeine
	PDE3 inhibitor	Amrinon, milrinone
	PDE4 inhibitor	Papaverine
	PDE5 inhibitor	Sildenafil
	HDAC inhibitor	Valproic acid
	HDAC3/HDAC7 inhibitor	Carbamezepine
Glycosidases (viral)	α-glycosidase inhibitor	Zanamivir, oseltamivir
Glycosidases (human)	α-glycosidase inhibitor	Acarbose
Lipases	Gastrointestinal lipases inhibitor	Orlistat
Phosphatases	Calcineurin inhibitor	Cyclosporin
	Inositol polyphosphate phosphatase inhibitor	Lithium ions
GTPases	Rac1 inhibitor	6-Thio-GTP (azathioprine metabolite)
Phosphorylases	Bacterial C55-lipid phosphate dephosphorylase inhibitor	Bacitracin
Lyases		
DOPA decarboxylase	Inhibitor	Carbidopa
Carbonic anhydrase	Inhibitor	Acetazolamide
Histidine decarboxylase	Inhibitor	Tritoqualine
Ornithine decarboxylase	Inhibitor	Eflornithine
Soluble guanylyl cyclase	Activator	Nitric acid esters, molsidomine

Type	Activity of drug	Drug examples
Isomerases		
Alanine racemase	Inhibitor	D-Cycloserine
DNA gyrases (bacterial)	Inhibitor	Fluoroquinolones
Topoisomerases	Topoisomerase I inhibitor	Irinotecan
	Topoisomerase II inhibitor	Etoposide
Δ8,7 isomerase (fungal)	Inhibitor	Amorolfin
Ligases (known as synthases)		
Dihydropteroate synthase	Inhibitor	Sulphonamides
Thymidylate synthase (fungal and human)	Inhibitor	Fluorouracil
Thymidylate synthase (human)	Inhibitor	Methotrexate, pemetrexed
Phosphofructokinase	Inhibitor	Antimony compounds
mTOR	Inhibitor	Rapamycin
Haem polymerase (Plasmodium)	Inhibitor	Quinoline antimalarials
1,3-β-D-glucansynthase (fungi)	Inhibitor	Caspofungin
Glucosylceramide synthase	Inhibitor	Miglustat

3.10.3. Enzymes Are a Distinct Target Class

Enzymes are biocatalysts. Among all other catalytic mechanisms shown by enzymes as was discussed earlier under the heading of “mechanism of enzyme catalysis” in this module, they ability of formation and break down of specific covalent chemical bonds makes enzyme a unique drug target and different from, Cell surface receptors, ion channels, transporters, nuclear hormone receptors, and nucleic acid/ribosome target. In drug discovery, the focus is always on the binding event of the drug with the enzyme target. Enzyme catalysis progresses through binding events, conformational changes, one or more transition states, or reaction intermediates, and product release, and all of these steps occur with defined rate constants. The rate constants define a thermodynamic profile that can be used for drug design, and this differentiates enzymes from all other target classes.

Drugs regulate the rate of chemical reactions of an enzyme. Many of most important and powerful modern drugs act on enzymes in the plasma or inside cells. Their action is usually via enzyme blockade and examples include the

angiotensin I-converting enzyme (ACE) inhibitors. Almost all biological reactions are carried out under catalytic influence of enzymes which are the major drug target. Drug may increase (stimulate) or decrease the activity of enzymes. However, enzyme stimulation is less common by drug. Generally, drugs inhibit the enzyme which is the common mode of action of drug and is the best approach for a drug design. We have passed through several kinds of enzyme inhibition and accordingly drugs are designed. There are mainly the following types of drugs which target enzyme.

1. **Enzyme Substrate Analogues/Inhibitor as Enzyme-Targeted Drugs**
2. **Transition-State Inhibitors as Enzyme-Targeted Drugs**
3. **Irreversible Inhibitors as Enzyme-Targeted Drugs:** The following 25 enzymes are irreversibly inhibited by drugs.

   (1) Serine Type D-Ala-D-Ala Carboxypeptidase; (2) β-Lactamase; (3) Acetylcholinesterase; (4) UDP-*N*-acetylglucosamine 1-Carboxyvinyltransferase (Fosfomycin); (5) Prostaglandin-Endoperoxide Synthase (Aspirin); (6) Unspecific Monooxygenase; (7) Amine Oxidase (Flavin-Containing); (8) Thymidylate Synthase; (9) Ornithine Decarboxylase; (10) Alanine Racemase; (11) H^+/K^+ ATPase; (12) Triacylglycerol Lipase; (13) Ribonucleoside-Diphosphate Reductase; (14) Iodide Peroxidase; (15) Thyroxine 5'-Deiodinase; (16) Aldehyde Dehydrogenase; (17) Thrombin; (18) Factor Xa; (19) 4-Hydroxyphenylpyruvate Dioxygenase; (20) Vitamin K Epoxide Reductase; (21) Ile tRNA Synthetase; (22) DNA-Directed DNA Polymerase; (23) 3-Oxo-5-α-steroid 4-Dehydrogenase; (24) Enoyl-Acyl Carrier Protein Reductase; (25) Xanthine Oxidase.

4. **Reaction Intermediate Traps as Enzyme-Targeted Drugs**
5. **Boronic Acid-Based Inhibitors as Enzyme-Targeted Drugs**
6. **Noncompetitive Inhibitors as Enzyme-Targeted Drugs**
7. **Activators as Enzyme-Targeted Drugs**

3.10.3.1. Enzyme Substrate Analogues/Inhibitor as Enzyme-Targeted Drugs

The majority of marketed enzyme-targeted drugs are related to enzyme substrate structure. These drugs, either undergo catalysis in the active site of an enzyme, chemically react with an enzyme cofactor, or contain a structural motif related to the substrate. Antibiotics, including penicillins, cephalosporins, and carbenapems, that target serine type D-Ala-D-Ala carboxypeptidase, and they all bear some structural similarity to the terminal D-Ala-D-Ala of bacterial peptidoglycan. In addition, they all undergo catalysis on the enzyme and acylate the active site serine. Similarly, inhibitors of β-lactamase, are used to overcome β-lactamase resistance by acylating the β-lactamase active site serine.

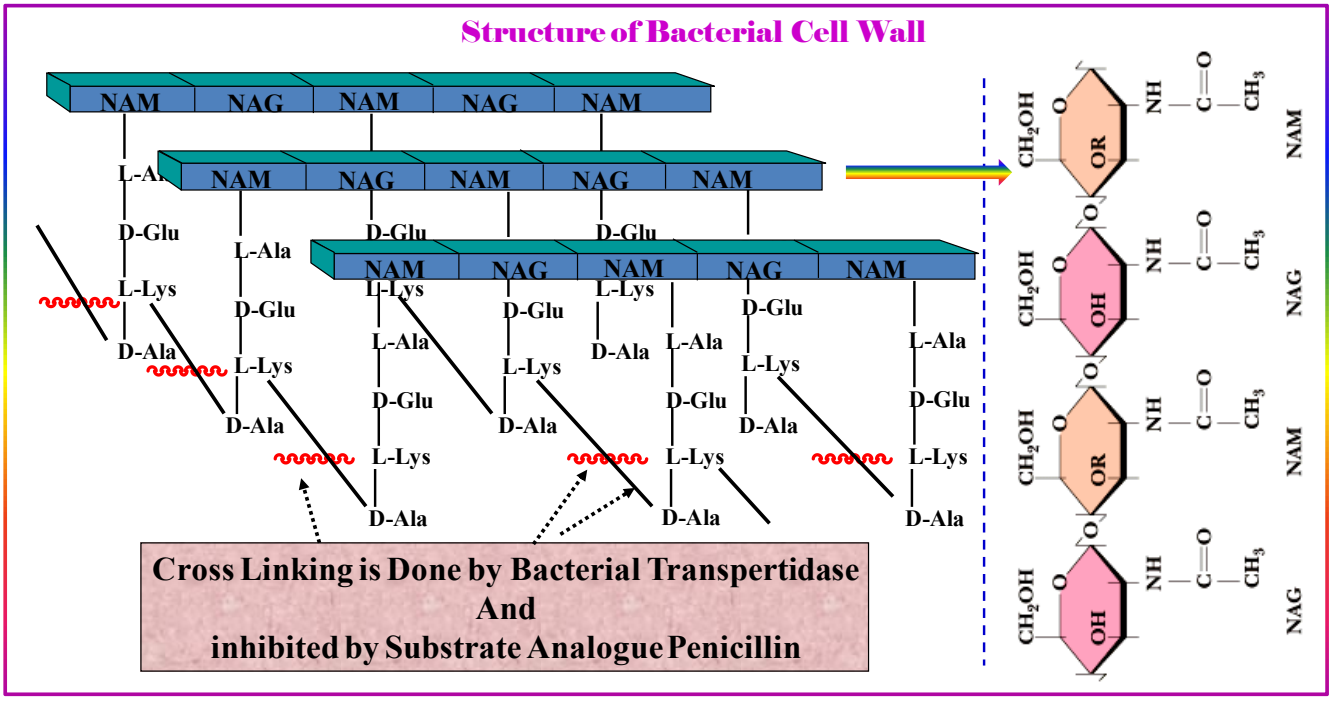


Figure 3.6: Structure of Bacterial cell wall.

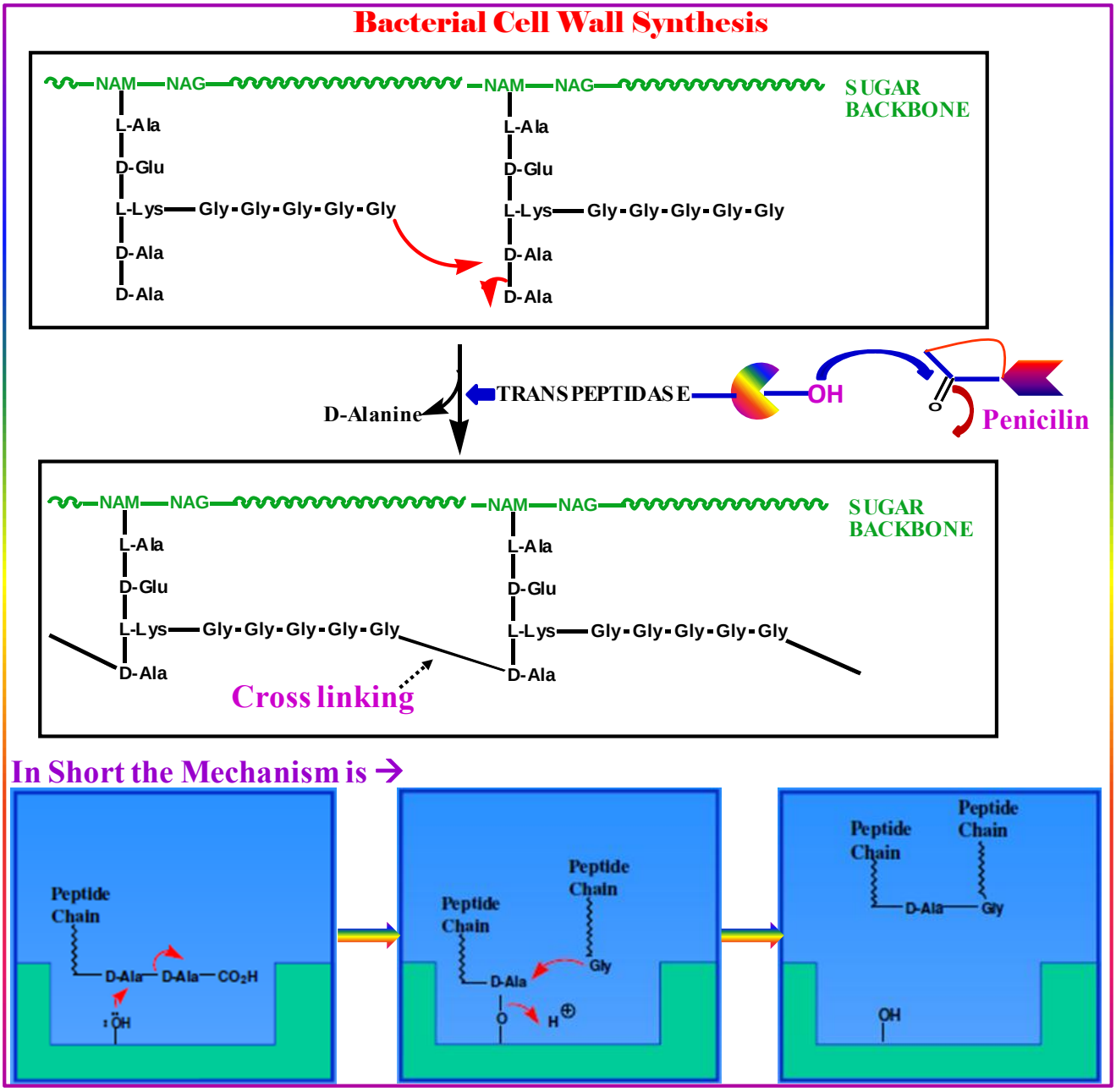


Figure 3.7: Mechanism of Bacterial cell wall synthesis.

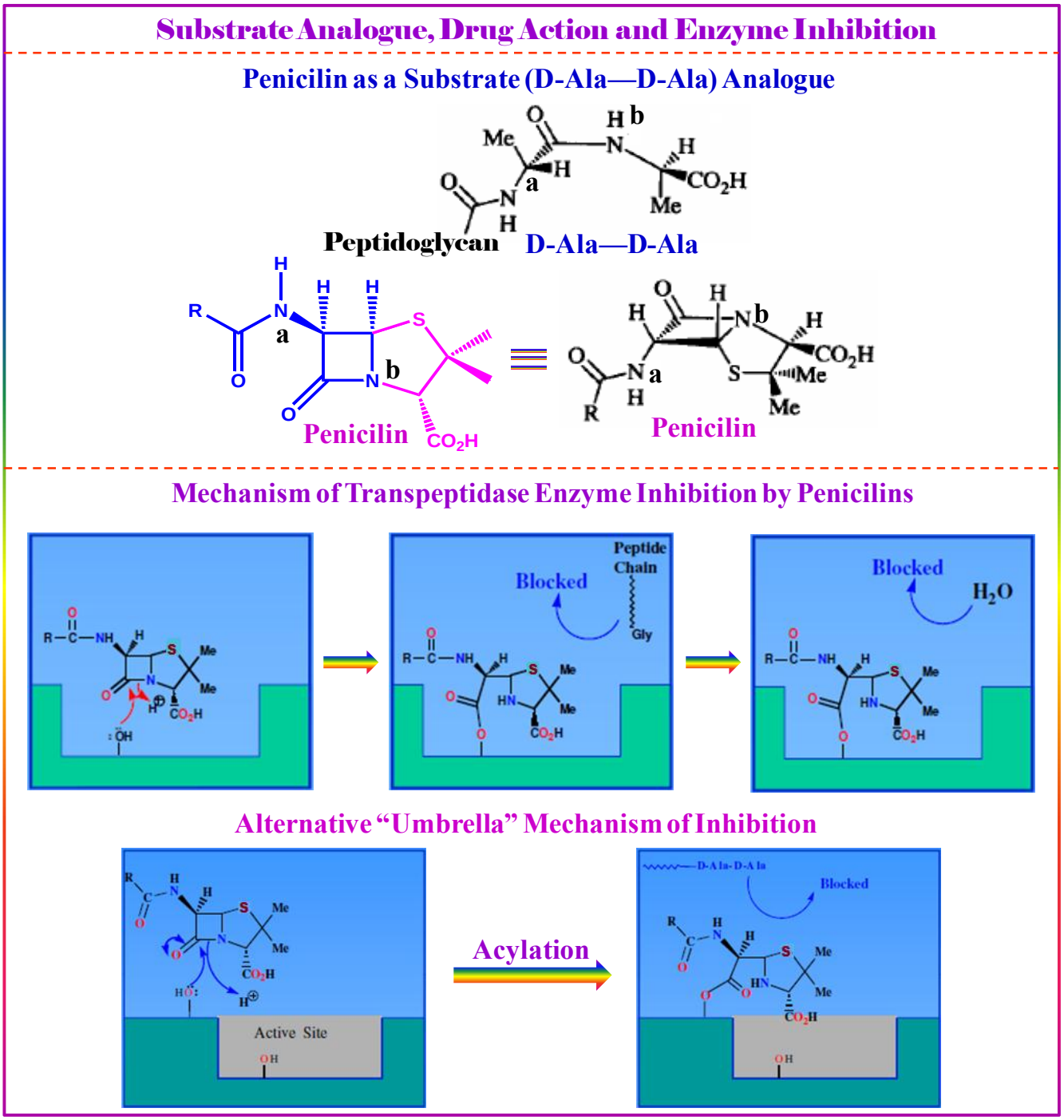


Figure 3.8: Mechanism of Bacterial transpeptidase inhibition by penicilin-an enzyme substrate analogue.

The sulfonamide inhibitors of dihydropteroate synthase are another example. The sulfonamides are structural analogues of *p*-aminobenzoic acid, the substrate of dihydropteroate synthase, and act as competitive inhibitors of PABA.

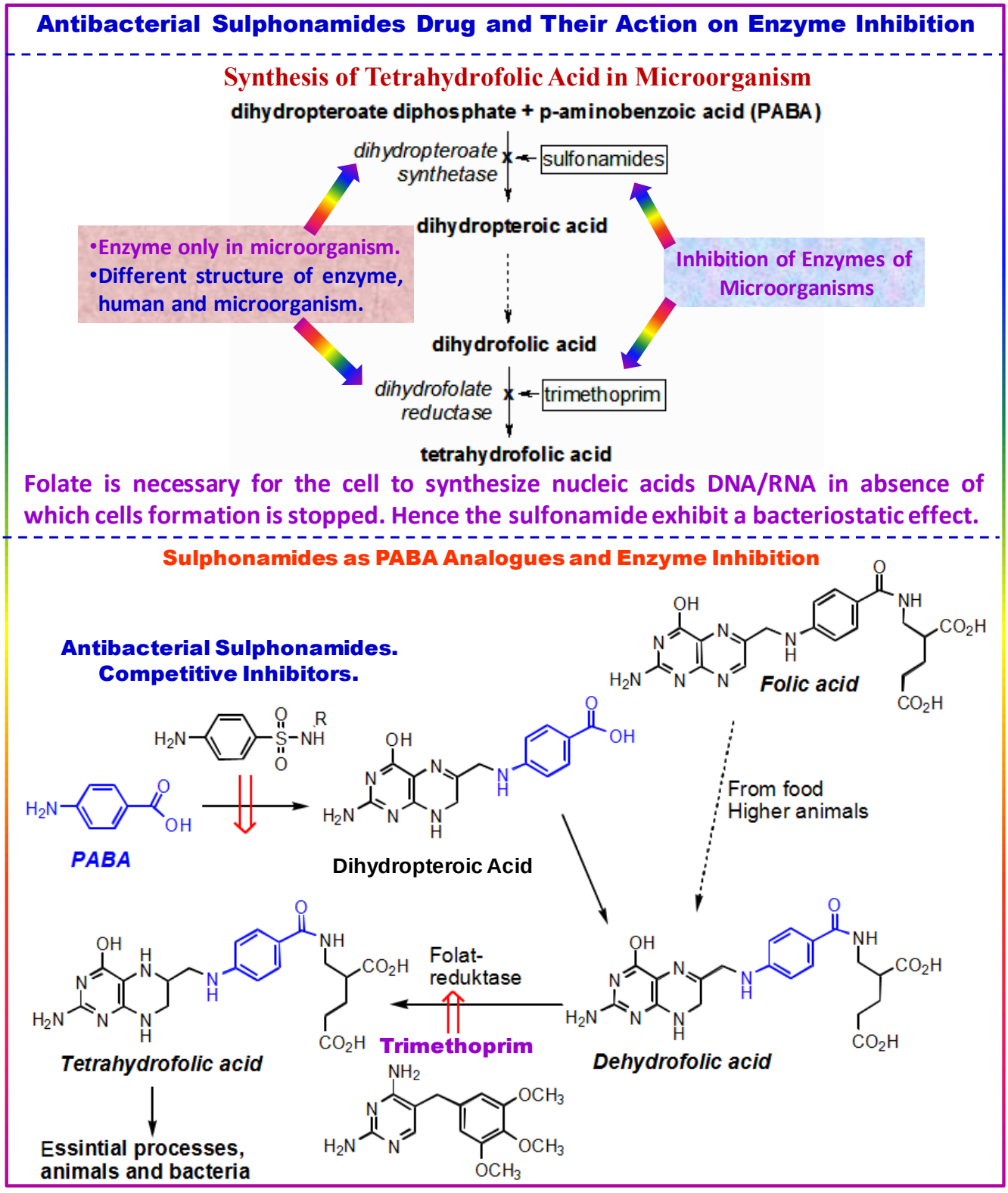


Figure 3.9: Mechanism of action of antibacterial sulphonamide drugs as PABA analogue.

3.10.3.2. Transition-State Inhibitors as Enzyme-Targeted Drugs

Transition State Stabilization by Enzymes: A catalyst bind the altered substrate in the transition state (T.S.) more tightly than it binds the substrate in the ground state (G.S.) whereupon lowering of the energy barrier of a reaction takes place. In the moment, lasting perhaps 1 msec, during which the catalytic event occurs, binding is enhanced by a factor that equals or surpasses the factor by which the catalyst enhances the rate of the reaction.

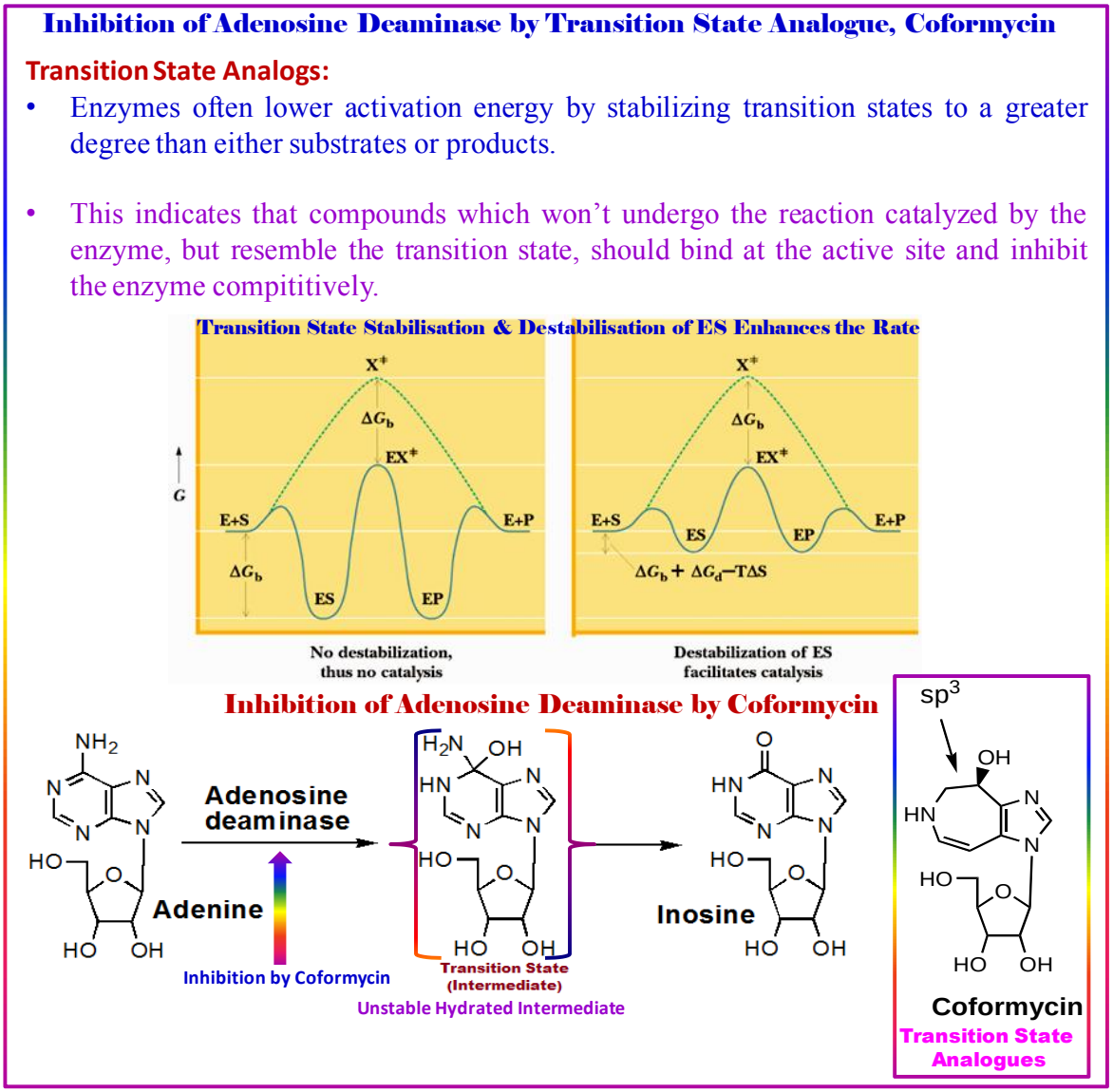


Figure 3.10: Mechanism of inhibition of adenosine deaminase by T.S. analogue Coformycin.

The transition-state analogues function as tight binding enzyme inhibitors. Till 1980s, most of the known examples of transition state analogues were natural products. Later on, in the 1990s, several synthetic inhibitors became the predominate examples of transition-state inhibitors. As for example, hydrolytic deamination of adenosine to inosine, catalyzed by fungal and mammalian enzymes (deaminase), is strongly inhibited by analogues of an Unstable Hydrated Intermediate formed by 1,6-addition of substrate water approaching from the front side of the adenosine ring. Thus, 1,6-hydroxymethyl-1,6-dihydropurine ribonucleoside (HDHPR) and the antibiotics coformycin and 2'-deoxycoformycin are powerful competitive inhibitors whose structures are very similar in structure to the postulated intermediate in the catalytic process.

Captopril, the first rationally designed enzyme-targeted drug, can be considered a transition-state inhibitor of peptidyl-dipeptidase A, also known as ACE. For HIV retropepsin (HIV protease), a variety of structure-assisted drug design techniques were successful in developing inhibitors. Saquinavir, the first marketed HIV retropepsin inhibitor, involved hydroxyethylamine isosteres that function as transition-state analogues.

HIV-1 Protease Inhibition: Transition State Analogue

HIV-1 Protease*: A novel aspartic protease*

- HIV-1 protease cleaves the polyprotein of the HIV genome to produce functional proteins.
- This is a remarkable imitation of mammalian aspartic proteases.
- HIV-1 protease is a homodimer - more genetically economical for the virus.
- Active site is two-fold symmetric .
- Two Asp residues - one high pK_a, one low pK_a.

Therapy for HIV? *Protease inhibitors as AIDS drugs*

- If the HIV-1 protease can be selectively inhibited, then new HIV particles cannot form.
- Several novel protease inhibitors are currently marketed as AIDS drugs.
- A successful drug must be able to kill the virus in a human subject without blocking other essential proteases in the body.
- **Potent inhibitors are transition state inhibitors like Squinavir.**

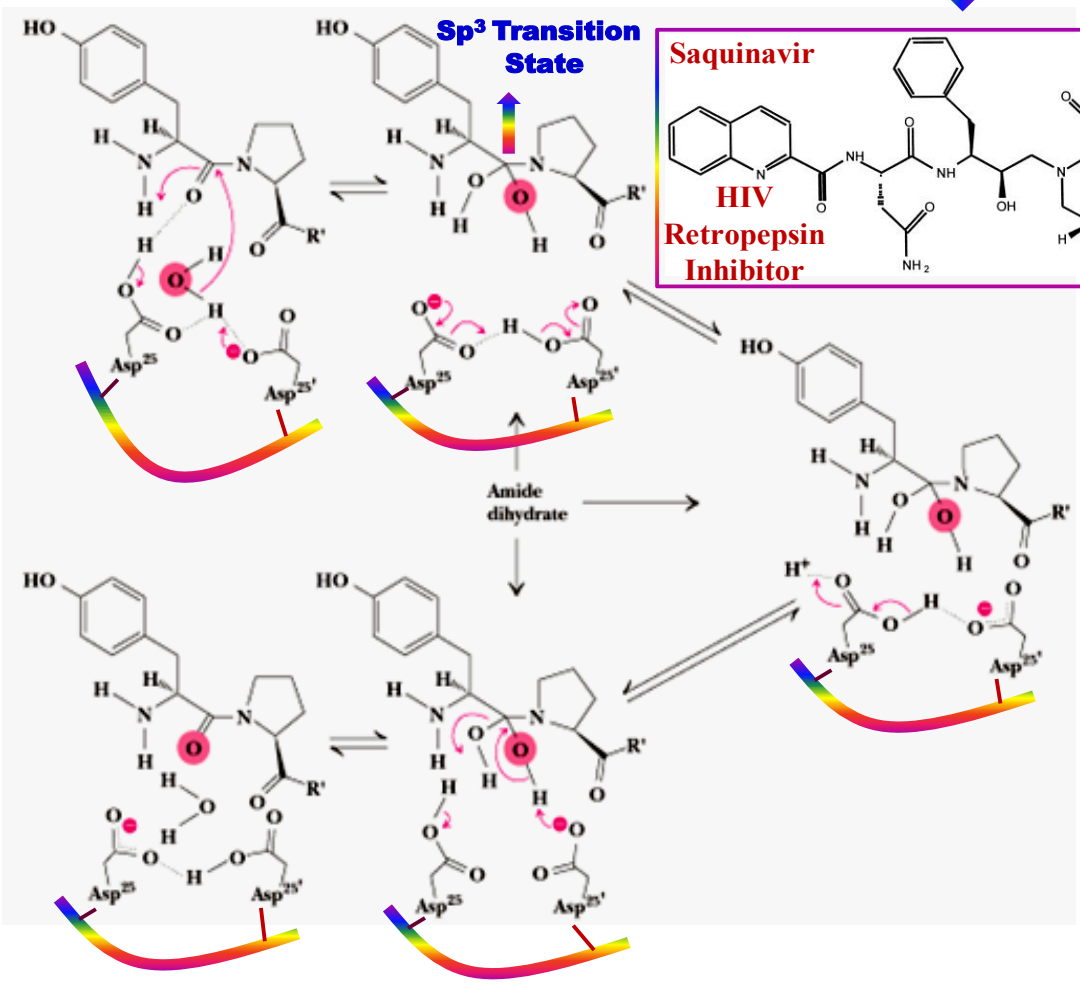


Figure 3.11: HIV-protease-I inhibition-a T.S. analogue approach.

3.10.3.3. Irreversible Inhibitors as Enzyme-Targeted Drugs

Drug design and discovery programs never set out to make irreversible inhibitors. Most of the enzymes that are irreversibly inhibited by drugs are covalently modified by the respective drug. In other cases, binding is so tight that the inhibitor remains bound for hours or even days, and binding can be considered functionally irreversible.

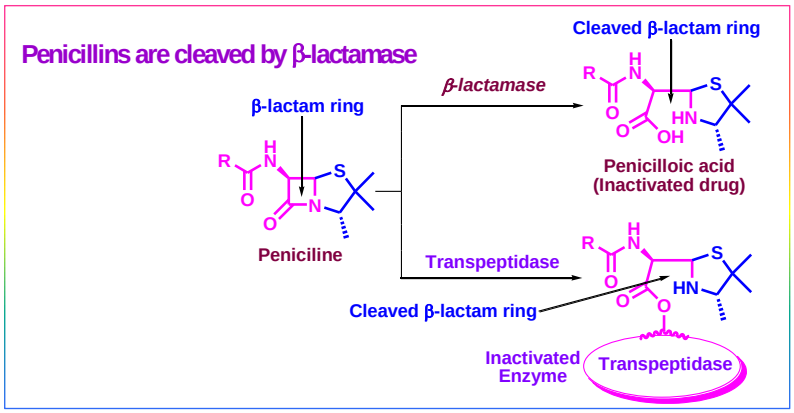
Irreversible Enzyme Inhibitors: β-Lactam Antibiotic

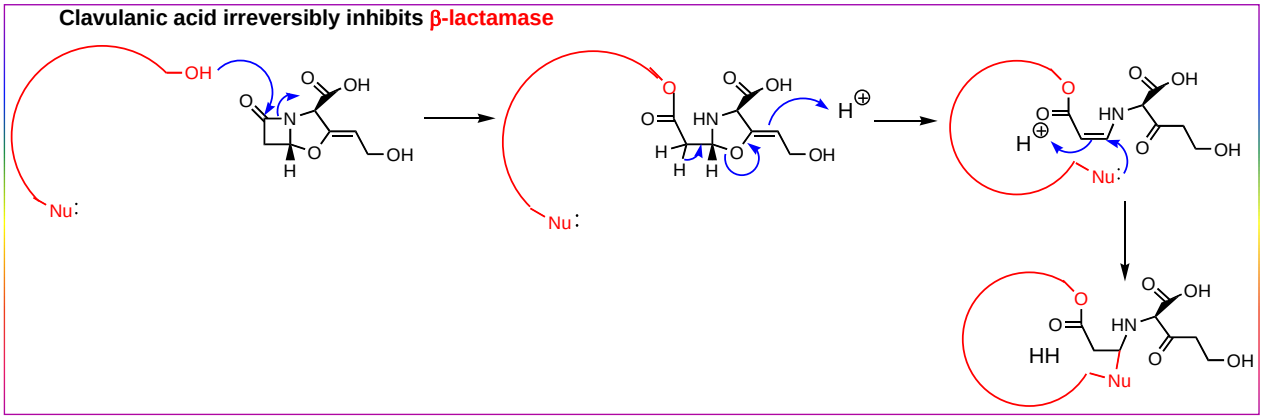
E + I ⟶ [E-I] ⟶ No further reaction

- Often covalent bonds between E and I
- Enzyme is permanently modified and inactivated.
- *Substrate may behaves as an inhibitor!*
- *Suicide substrate inhibitors or Mouse trap inhibitors or Trojan horse inhibitors*

Example:

1. **Affinity labels and active site directed irreversible inhibitors**
   - Structural recemblance with substrate
   - Electrophilic - alkylate nucleophilic subst in enzyme active site
   - Low selectivity - generally highly toxic
2. **Mechanism based irreversible enzyme inactivators**
   - Suicide substrate - kcat inhibitors - Trojan horse inhib. - latent alkylating agent ≈ Pro-drug, must be activated by the enzyme. Example Clavulanic Acid.





Serine type D-Ala-D-Ala carboxypeptidase is the most thoroughly studied example of a covalently inactivated enzyme drug target. All β-lactam antibiotics acylate the active site serine of the carboxypeptidase. This is generally a stable acylation that is resistant to hydrolysis, and therefore, it effectively eliminates all transpeptidase activity in the bacterium.

The resistance to β-lactam antibiotics occurs via hydrolysis of β-lactams by β-lactamase. While the acyl−enzyme intermediate in D-Ala-D-Ala carboxypeptidase is stable, the β-lactamase acyl−enzyme intermediate hydrolyzes at rates of 100−4000 s^{-1}. However, several naturally occurring β-lactams, including clavulanate, tazobactam, and sulbactam, form kinetically stable acyl−enzyme intermediates and inactivate β-lactamase. These drugs are used to overcome β-lactamase resistance.

Inhibitors of AmpC Beta Lactamase

Due to widespread resistance, inhibitors of beta-lactamases are sought. Clavulanic acid (d) is one inhibitor; ceftazidime (b) is a beta-lactam that is resistant to beta-lactamases. New substrate analogs "c" are found to inhibit new broad spectrum beta-lactamases. All have similar structure: resistance to these are also anticipated.

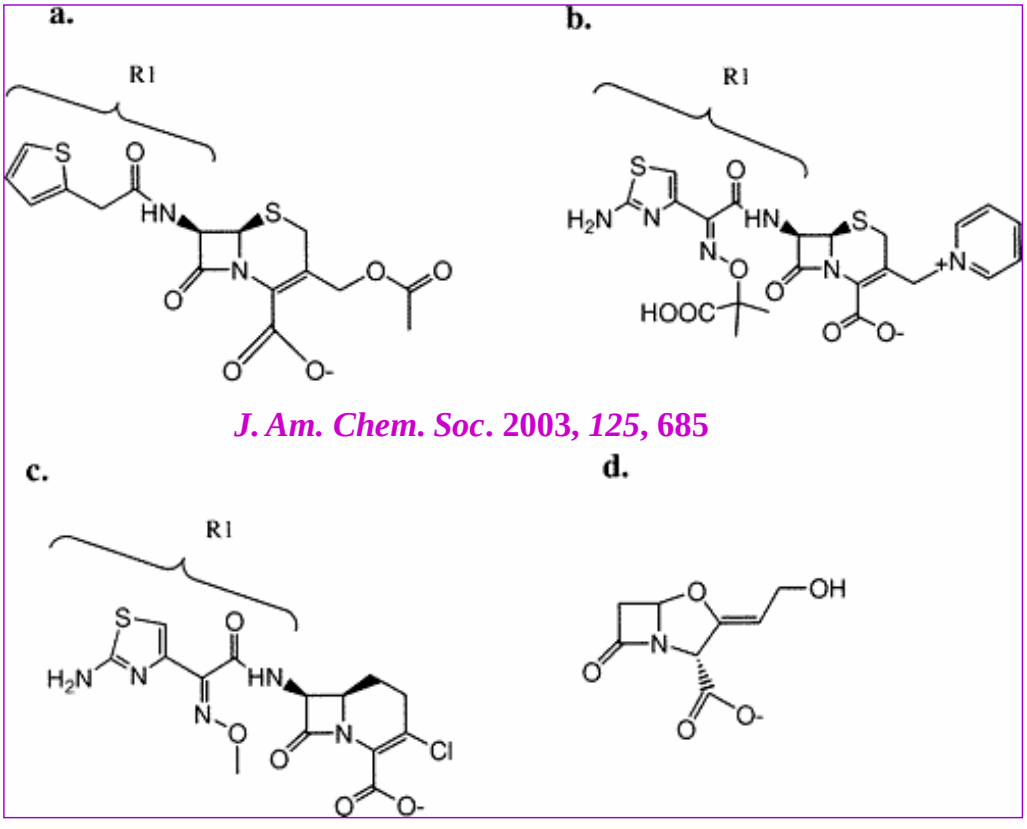


Figure 3.12: Some examples of β-lactamase inhibitor-irreversible inhiition.

Aspirin is another example of an irreversible covalent inactivator of prostaglandin-*endo*peroxide synthase, also called cyclooxygenase, or COX.

The DNA-polymerases is an important drug targets because of their critical role in replication. Deoxynucleotide-based inhibitors are recognized as substrates for the enzyme, and terminate strand extension because they lack the ribose hydroxyl necessary for ligation to the next nucleotide. In this sense, the inhibition is of irreversible types. Acyclovir, which targets herpes virus DNA-directed DNA polymerase, does produce irreversible enzyme inactivation in addition to nucleotide incorporation and strand termination.

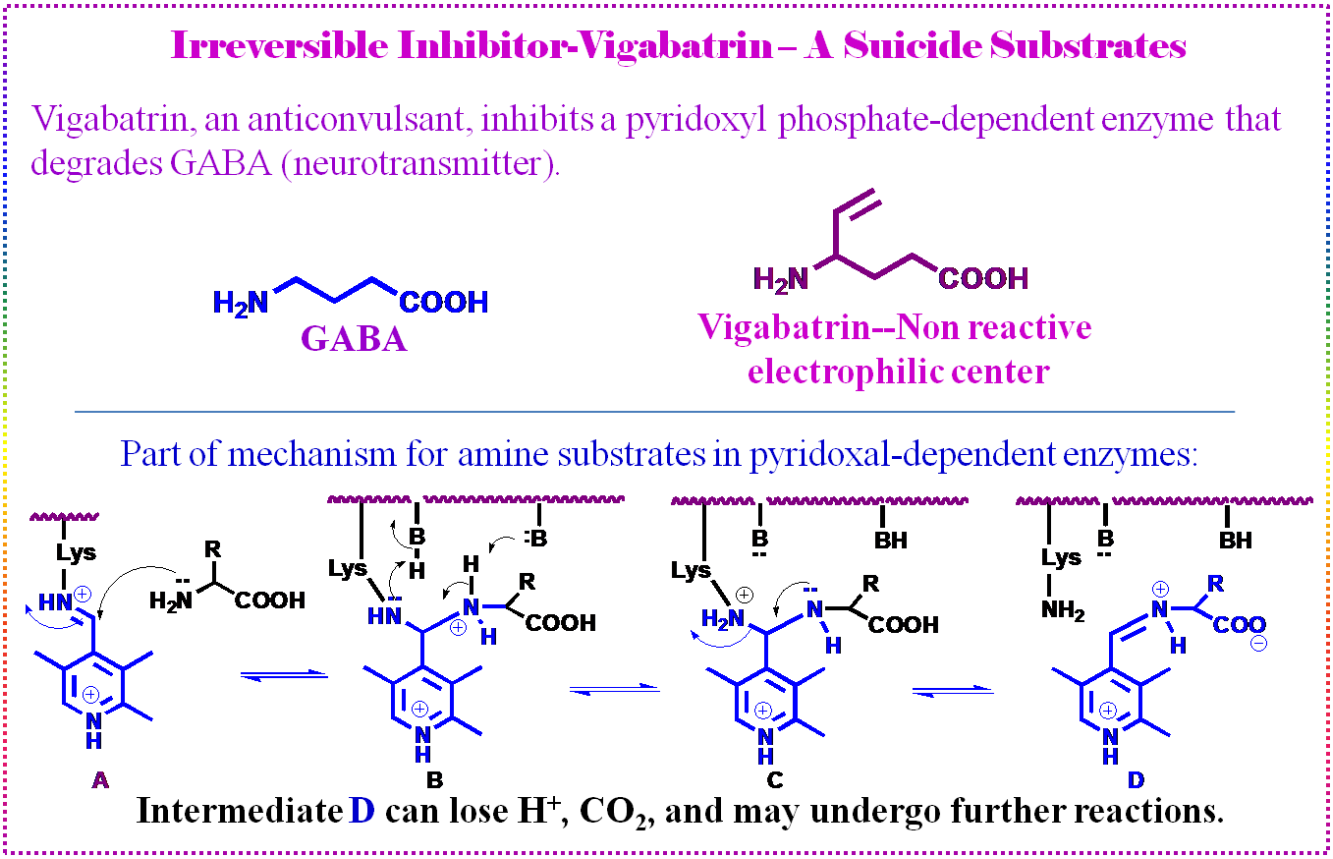


Figure 3.13a: Vigabatrin-a suicide substrate of GABA.

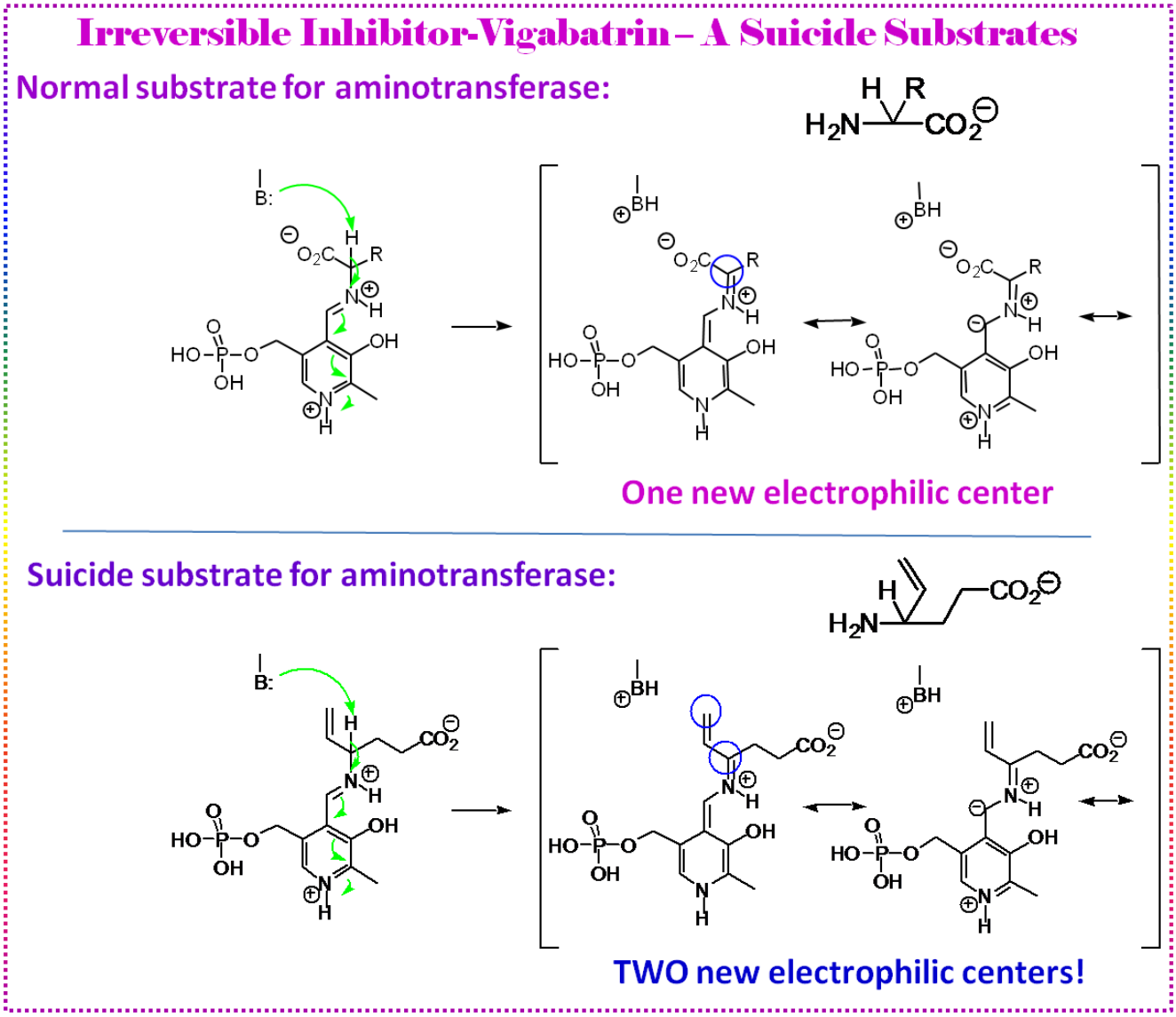


Figure 3.13b: Vigabatrin-a suicide substrate of GABA-Mechanism of action.

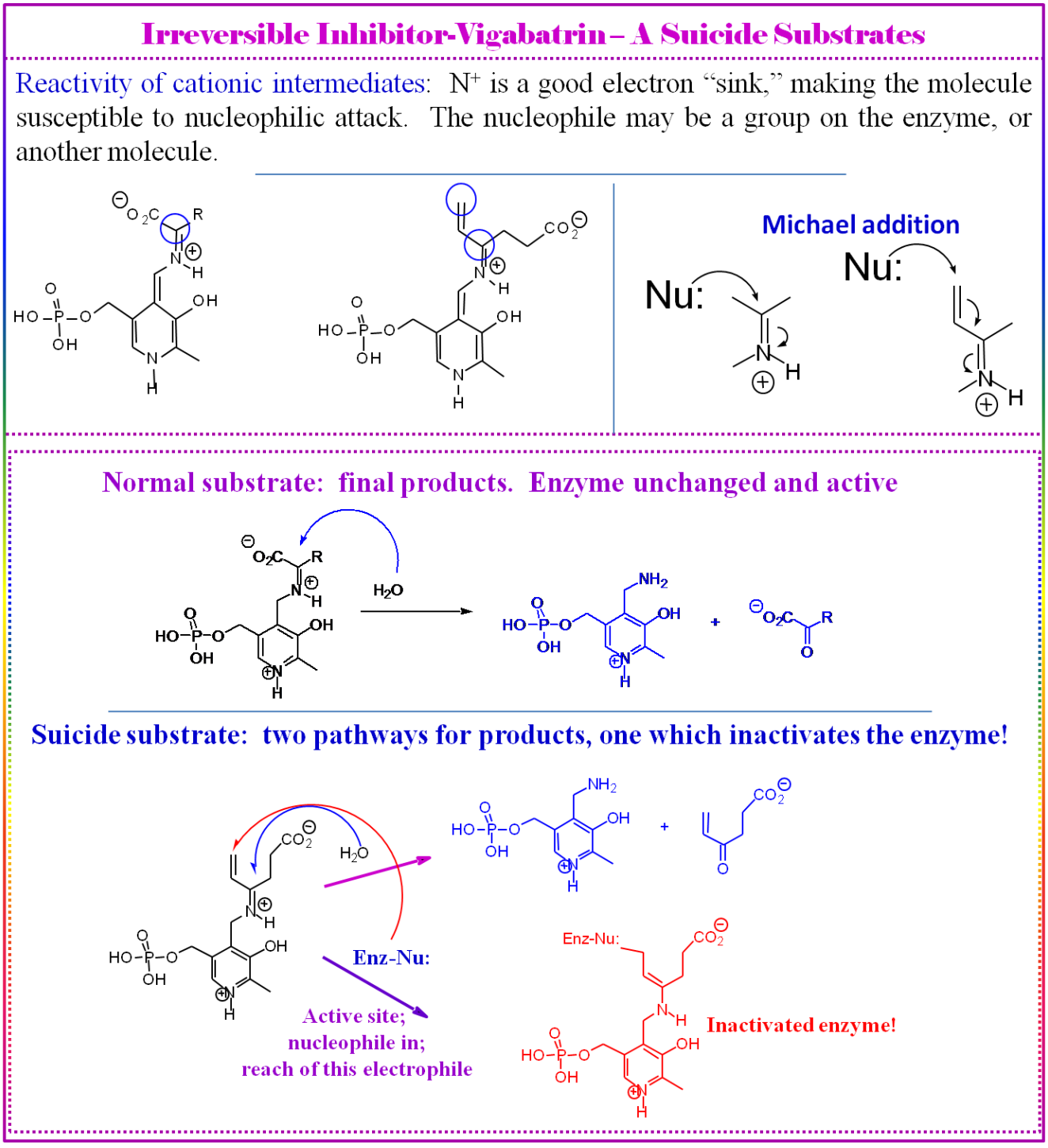


Figure 3.13c: Vigabatrin-a suicide substrate of GABA-Mechanism of action of enzyme inactivation.

3.10.3.4. Reaction Intermediate Traps as Enzyme-Targeted Drugs

The trapping of reaction intermediate is another mechanism for the formation of covalent inhibitory complexes between the enzyme and inhibitor. Inosine monophosphate (IMP) dehydrogenase and DNA topoisomerase fall in this category. Inosine monophosphate dehydrogenase catalyzes the oxidation of IMP to XMP through the formation of a covalent cystinyl intermediate at C-2 of the purine ring. Mycophenolic acid, an uncompetitive inhibitor, binds in the NAD cofactor site, and prevents the hydrolysis reaction, thus trapping and stabilizing the covalent E−XMP complex. The structure of the trapped complex shows that the bicyclic ring system of mycophenolic acid packs underneath the hypoxanthine ring of XMP, thereby preventing release of the intermediate.

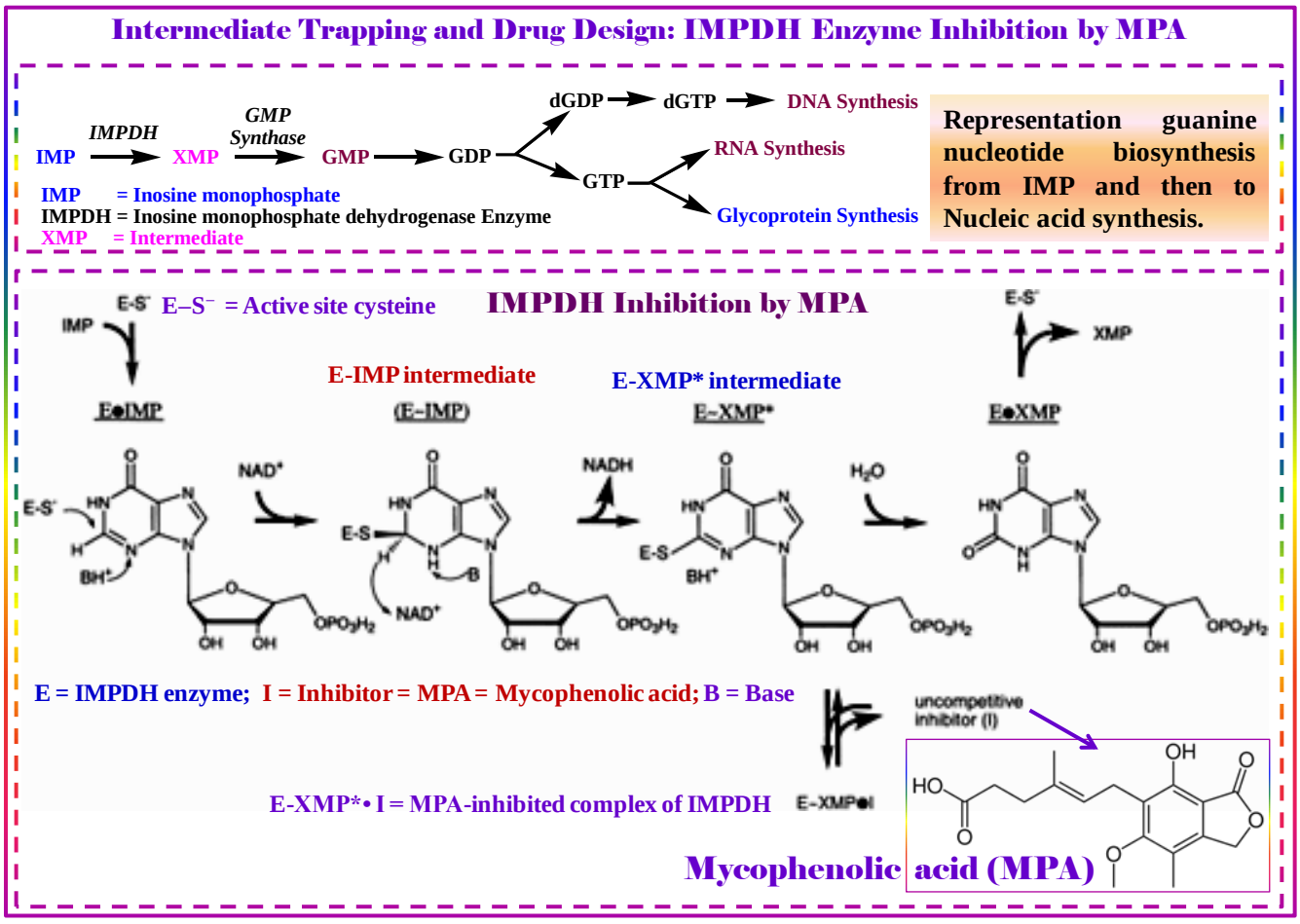


Figure 3.14: IMPDH enzyme inhibition by MPA-an example of intermediate trapping.

Mammalian and bacterial DNA topoisomerases catalyze the topological rearrangement of supercoiled and concatenated DNA, and serve as targets for the antitumor agents topotecan and irinotecan, and as targets for the fluoroquinoline antibiotics. In human topoisomerase I, the enzyme catalyzes single-strand cleavage and formation of a phosphodiester bond between Tyr^{723} and the DNA 3‘-phosphate. Biochemical evidence indicates that camptothecin, the parent compound for the marketed drugs irinotecan and topotecan, stabilizes the covalent complex, and several models suggest that it binds near the DNA cleavage site. In topoisomerase II, the enzyme cuts two DNA strands and generates four free ends. The enzyme covalently links both of the 5'-ends to catalytic residues in the active site, in effect prying open the DNA on the ends of a molecular fork and opening a gate through the DNA. The quinolone antibiotics are thought to stabilize this covalent intermediate.

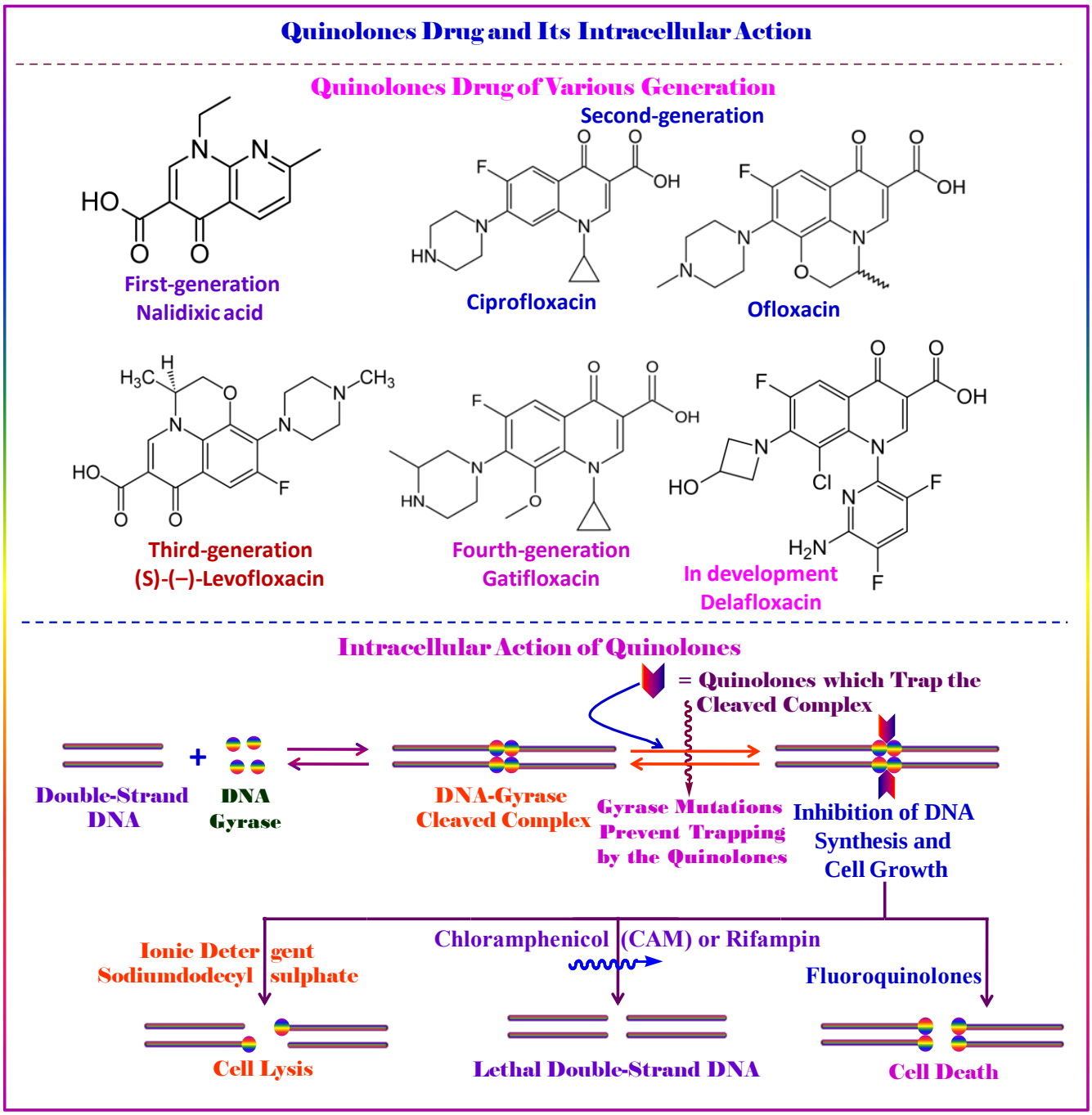


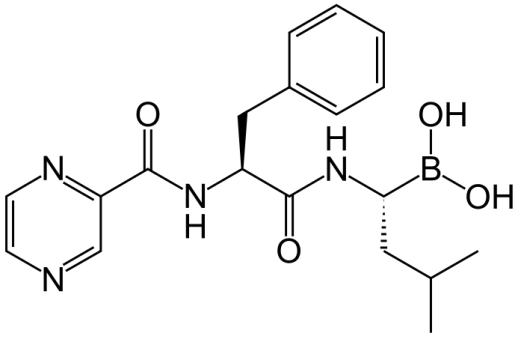
Figure 3.15: Various quinolone drugs and their mechanism of intracellular action.

3.10.3.5 Boronic Acid-Based Inhibitors as Enzyme-Targeted Drugs

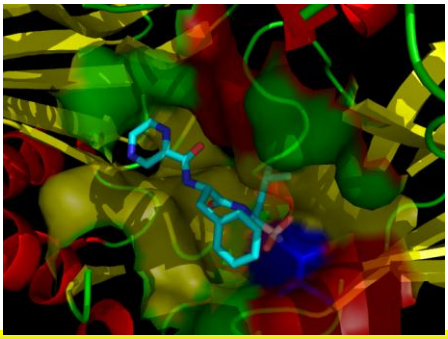
Boronic acids act as strong Lewis acids. The ready interconversion of a trigonal planar sp^2 geometry to a tetrahedral sp^3 geometry by substitution at boron is remarkably similar to the formation of tetrahedral intermediates in many hydrolytic enzymes, and allows boronic acids to act as reaction intermediate analogues with potent inhibitory properties. The first FDA-approved boronic acid-based inhibitor which came into the market in 2003 is Bortezomib which inhibits proteasome degradation of intracellular proteins and has shown efficacy in solid and hematological cancers. This is a slow-binding inhibitor.

Boronic Acid-Based Inhibitors as Enzyme-Targeted Drugs

Bortezomib: Inhibition of Proteasome Degradation of Intracellular Proteins



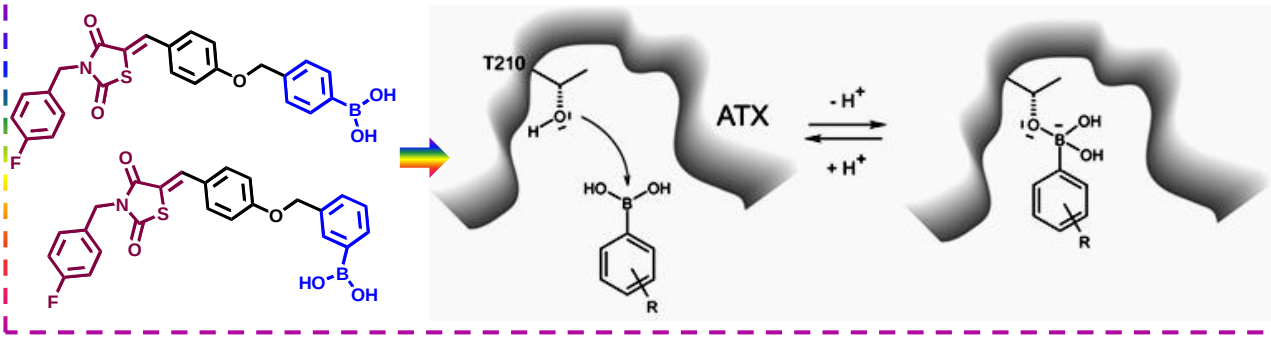
Structure of Bortezomib
The drug is a tripeptide and can be written as Pyz-Phe-boroLeu, (Pyz = pyrazinoic acid).



Bortezomib bound to the core in a yeast proteasome. The bortezomib molecule is in the center, surrounded by the local protein surface. The blue patch is catalytic threonine residue whose activity is blocked by the presence of bortezomib.

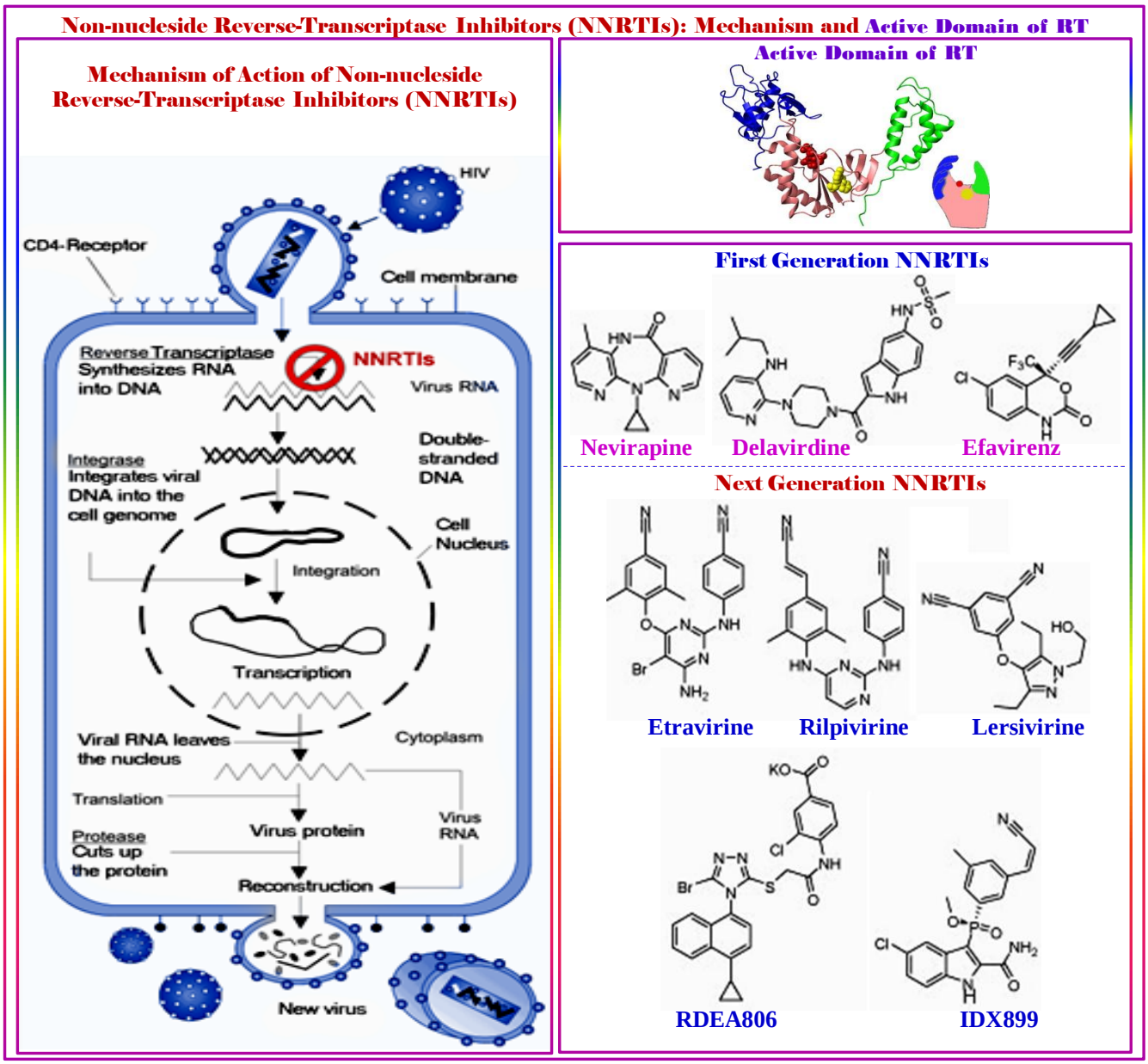
Boronic Acid Inhibitors of Autotaxin (ATX)

Autotaxin (ATX) is an extracellular enzyme that hydrolyses lysophosphatidylcholine (LPC) to produce the lipid mediator lysophosphatidic acid (LPA). The ATX−LPA signaling axis has been involved in various physiological and pathological processes such as, vascular development, inflammation, fibrotic disease, and tumor progression. Therefore, small molecule inhibitors is an attractive therapeutic strategy to target ATX. Huib Ovaa *et al.* have reported that 2,4-thiazolidinediones inhibit ATX activity and inhibitory potency was dramatically increased by introduction of a boronic acid moiety which was designed to target the active site threonine in ATX. The Boronic acid compounds show a mixed-type inhibition against ATX-mediated LPC hydrolysis in the nanomolar range. These Boronic acids bind to ATX in a reversible manner by binding to the T210 oxygen nucleophile of ATX.



3.10.3.6. Noncompetitive Inhibitors as Enzyme-Targeted Drugs

As we have seen the most enzyme-based drugs are active site inhibitors. However, noncompetitive inhibitors that do not bind in the active site may also show drug activity. Nonnucleoside reverse transcriptase inhibitors, including nevirapine, are a good example. Nonnucleoside inhibitors do not interfere with nucleotide binding or the change in conformation induced by nucleotide binding, but, they bind at an allosteric site of the enzyme, and thereby slowed down the rate of chemical catalysis. Therefore, the microscopic rate constants can provides a competitive advantage to enzymologists developing noncompetitive inhibitors as drugs. Two inhibitors might have the same K_i, but if one slows the rate of catalysis 10-fold more than the other, it will be a more potent drug.



3.10.3.7. Activators as Enzyme-Targeted Drugs

We know that drugs may activate or inactivate the enzyme activity. Till now we were discussing about inhibitors of pathogen's enzyme as the drug to give a beneficial clinical effect to a patient. However, there are no pure enzyme activators on the marketaccording to a pure k_{cat} or k_{cat}/K_m activator. However, recently, the concept of drug design target to develop *enzyme* activators (endogeneous enzyme) which might lead to a beneficial clinical effect in response to a certain disease and in future this approach may lead to a numbers of such kinds of drugs.

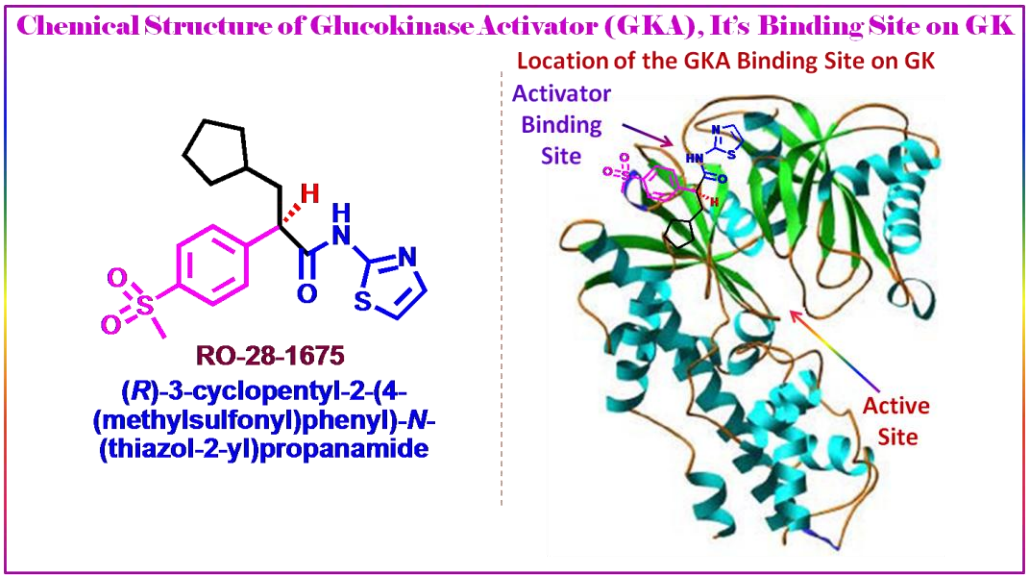


Figure 3.16: RO-28-1675 as glucokinase activator.

Joseph Grimsby *et al.*, of Roche have recently discovered activators of glucokinase that increase k_{cat} and decrease the $S_{0.5}$ for glucose, and these may offer a treatment for type II diabetes. Glucokinase (GK) plays a key role in whole-body glucose homeostasis by catalyzing the phosphorylation of glucose in cells that express this enzyme, such as pancreatic β cells and hepatocytes. By screening of a library of 120,000 structurally diverse synthetic compounds, they found one small molecule that increased the enzymatic activity of GK. Chemical optimization of this initial molecule led to the synthesis of RO-28-0450 as a lead GK activator which is a class of antidiabetic agents that act as nonessential, mixed-type GK activators (GKAs) that increase the glucose affinity and maximum velocity (V_{max}) of GK. RO-28-0450 is a racemic compound. Activation of GK was exquisitely sensitive to the chirality of the molecule: The R enantiomer, RO-28-1675, was found to be a potent GKA, whereas the S enantiomer, RO-28-1674, was inactive. RO-28-1675 also reversed the inhibitory action of the human glucokinase regulatory protein (GKRP). The activators binding in a glucokinase regulatory site originally was discovered in patients with persistent

hyperinsulinemic hypoglycemi. The result of RO-28-1675 as a potent small molecule GKA may shed light to the chemical biologists to devise strategy for developing activators. Thus for a success to this end we must focus on highly regulated enzymes, or cooperative enzymes such as glucokinase, where nature has provided binding sites that are designed to modulate catalysis.

3.11. Enzyme in Organic Synthesis

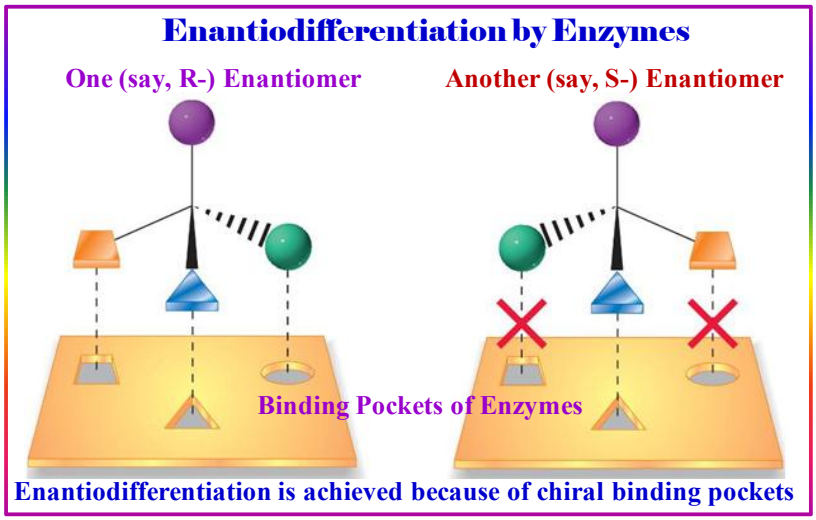
3.11.1. Introduction

Over the years the use of enzymes as catalysts for the preparation of novel organic molecules has received a steadily increasing amount of attention. Selectivity i.e. to obtain a high yield of a specific product is the prime importance of organic synthesis. There are a large number of selective organic reactions available for most synthetic needs. However, chiral synthesis is a challenging task and the organic chemists are now also struggling in this area, although a considerable progress in asymmetric synthesis has been achieved in recent years.

Since long back, the natural catalyst, the Enzymes are known to active in organic chemical transformation and treated as “green” catalyst. Synthesis of (R)-Mandelonitrile in 1908 is the first example ofenzyme in organic chemistry.

Increasing demand for optically pure compounds for applications in pharmaceuticals, cosmetics, food industry etc., since then Enzymes are being used in a number of biocatalytic application because of the following reasons.

- Enzymes are efficient catalysts
- Biocatalysis is Environmentally "green" and possess high safety profile
- Enzyme catalysis need mild reaction conditions (pH 5-8, 20-40 °C, pressure) which minimizes problems of undesired side-reactions such as decomposition, isomerization, racemization and rearrangement, which often plague traditional methodology.
- They Catalyze a broad spectrum of reactions.
- Biocatalysts can perform transformations that are difficult to emulate using more traditional organic chemistry.
- Enzymes are chiral catalysts and are often able to produce optically active molecules that can be used as building blocks for the preparation of other chiral molecules of biological importance.
- Most importanly, they offers three major types of selectivities: high Chemoselectivity, high Regioselectivity and diastereoselectivity, and Enantioselectivity/stereospecificity
- Biocatalysts save additional reaction steps compared to organic synthesis.
- Enantiodifferentiation is more important feature of enzyme.

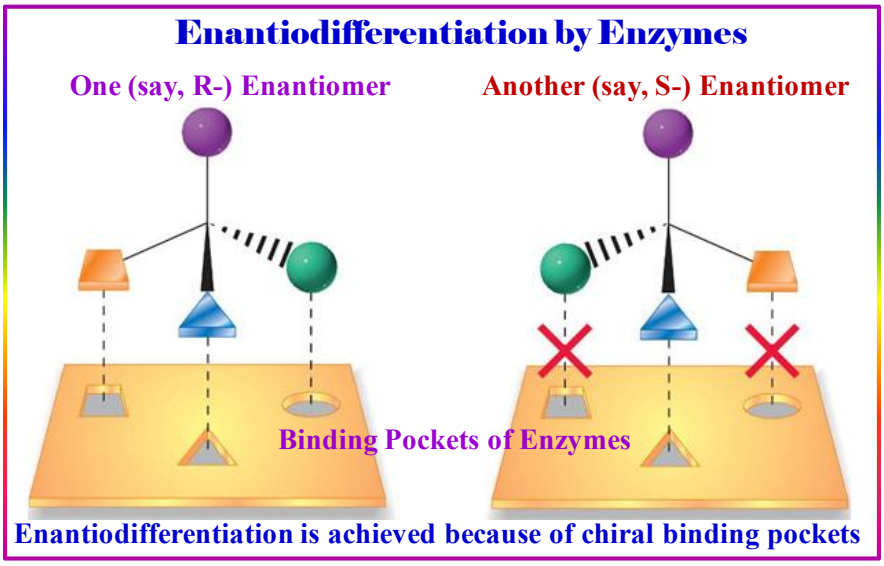


- Enzymes speed up the reaction because it binds more tightly to the transition state vs. ground state by factor approximately equal to rate acceleration.
- Enantioselectivity, in principle, has to do with the difference of energy in transition state $\Delta\Delta G$ which is the basis of "chiral recognition".

These reasons, especially the stereoselectivity and stereo differntiation, are the major reasons why synthetic chemists have become interested in biocatalysis. This interest in turn is mainly due to the need to synthesize enantiopure compounds as chiral building blocks for drugs.

Selectivity in Enzymes Originates From:

- The substrate bind to particular sites on enzymes which is often be the active site.
- The active site is comprised of a variety of chiral amino acid residues which give rise to a specific 3-D shape and chiral molecular features:
  - Charges: CO_2^- , NH_3^+, $=NH-^+$
  - Polar groups: OH, C=O, CONH
  - Hydrophobic groups: Ph, Alkyl, SMe
- In enzymes, reaction centres are also present:
  - Asp-His-Ser in esterases
  - SH in some proteases
  - Metal ions (CYP-450, iNOS).
- Small molecules bind to this active pocket of enezyme by a combination of:
  - Shape complementarity
  - Energetically favourable interactions-which leads to the enatiodiferentiation and selectivity in enzyme mediated organic transformation.



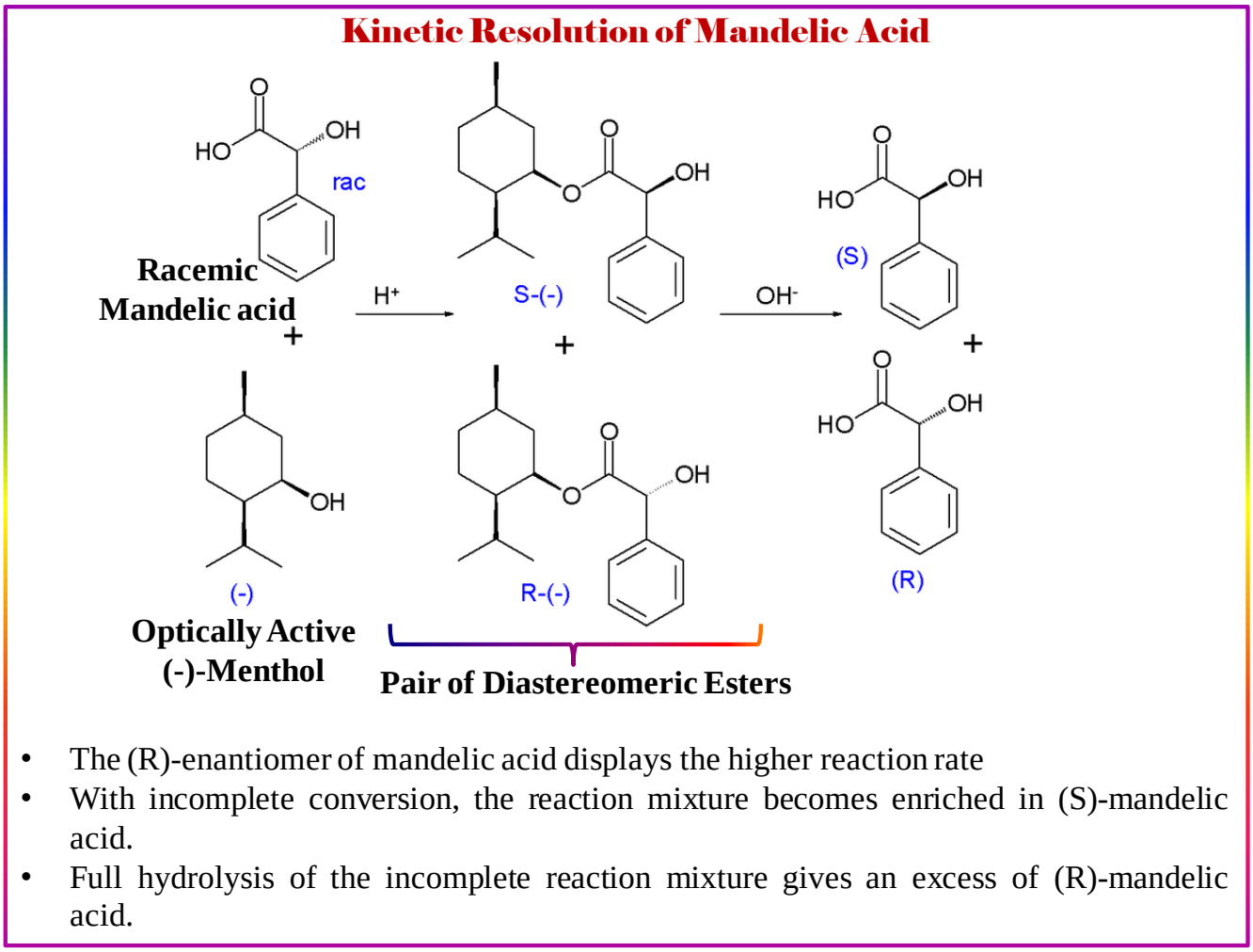
3.11.2. Classification of Enzymes

About 3,000 enzymes are known and 25,000 are speculated of which only 10% of known are commercially available. Two of the most popular areas of biotransformations that have a very great relevance to organic synthesis involve the hydrolysis reaction by the use of hydrolase enzymes (25% of known enzymes) and organic oxidation-reduction reaction catalyzed by the enzyme class oxidoreductase (65 % of known enzymes).

Enzyme Class	*Catalyzed Reaction*
1. Oxidoreductases **(Dehydrogenase/Oxygenases)**	**Oxidation-reduction:** Catalyze redox reactions, usually NAD is used as a coenzyme; Oxygenation of C-H, C-C, C=C bonds.
2. Transferases **(acylase, phosphorylase)**	**Transfer of functional group:** Catalyze transfer of molecular groups like aldehydic, ketonic, acyl, sugar, phosphoryl or methyl from one molecule to another.
3. Hydrolases **(esterase, lipase)**	**Hydrolytic reactions:** Catalyze bond cleavage by the introduction of water; Hydrolysis-formation of esters, amides, lactones, epoxides, nitriles, anhydrides.
4. Lyases **(a.k.a synthase - decarboxylase)**	**Group elimination (forming double bonds):** Cleavage of C-Y (C or heteroatom) bonds to form double bonds or rings (or reverse rxn.); Addition-elimination of small molecules on C=C, C=N, CO bonds.
5. Isomerases **(racemase, epimerase)**	**Isomerization reaction:** Catalyze reactions involving intramolecular rearrangements, Racemisation, epimerisations.
6. Ligases **(a.k.a. synthetase-DNA ligase)**	**Bond formation with a triphosphate cleavage:** Formation or cleavage of C-Y (C or heteroatom) bonds with triphosphate (ATP) hydrolysis; Formation-cleavage of C-O, C-S, C-N, C-C bonds with triphosphate cleavage.
7. Novel biocatalysts **(Engineered enzymes/ Evolved enzymes/ Catalytic antibodies/ Enzyme models and mimetics)**	**Various organic transformations**

3.11.2.1. Oxidoreductases: Use of Horse Liver Alcohol Dehydrogenase (HLADH)-Catalyzed Oxidations: Enzymatic Kinetic Resolution

In **kinetic resolution**, two enantiomers show different reaction rates in a chemical reaction, thereby creating an excess of the less reactive enantiomer. This excess goes through a maximum and disappears on full completion of the reaction. Because of the property of enantiodiferention shown by enzyme, they are being widely used for such purposes. Kinetic resolution was first observed by Marckwald and McKenzie in 1899 in the esterification reaction of racemic mandelic acid with optically active (-)-menthol to a pair of diastereomeric esters.



- The (R)-enantiomer of mandelic acid displays the higher reaction rate
- With incomplete conversion, the reaction mixture becomes enriched in (S)-mandelic acid.
- Full hydrolysis of the incomplete reaction mixture gives an excess of (R)-mandelic acid.

Figure 3.17: Kinetic resolution of Mandelic acid.

Enzymatic Kinetic Resolution: HLADH Enzyme:

- Oxidoreductases catalyse **oxidation and reduction reactions**
- They are dependent on NADH or NADPH **cofactor**
- Reaction involve in **recycling of cofactors**
- Can be used in **whole cell** systems
- the most useful for preparative applications is **dehydrogenases and reductases**

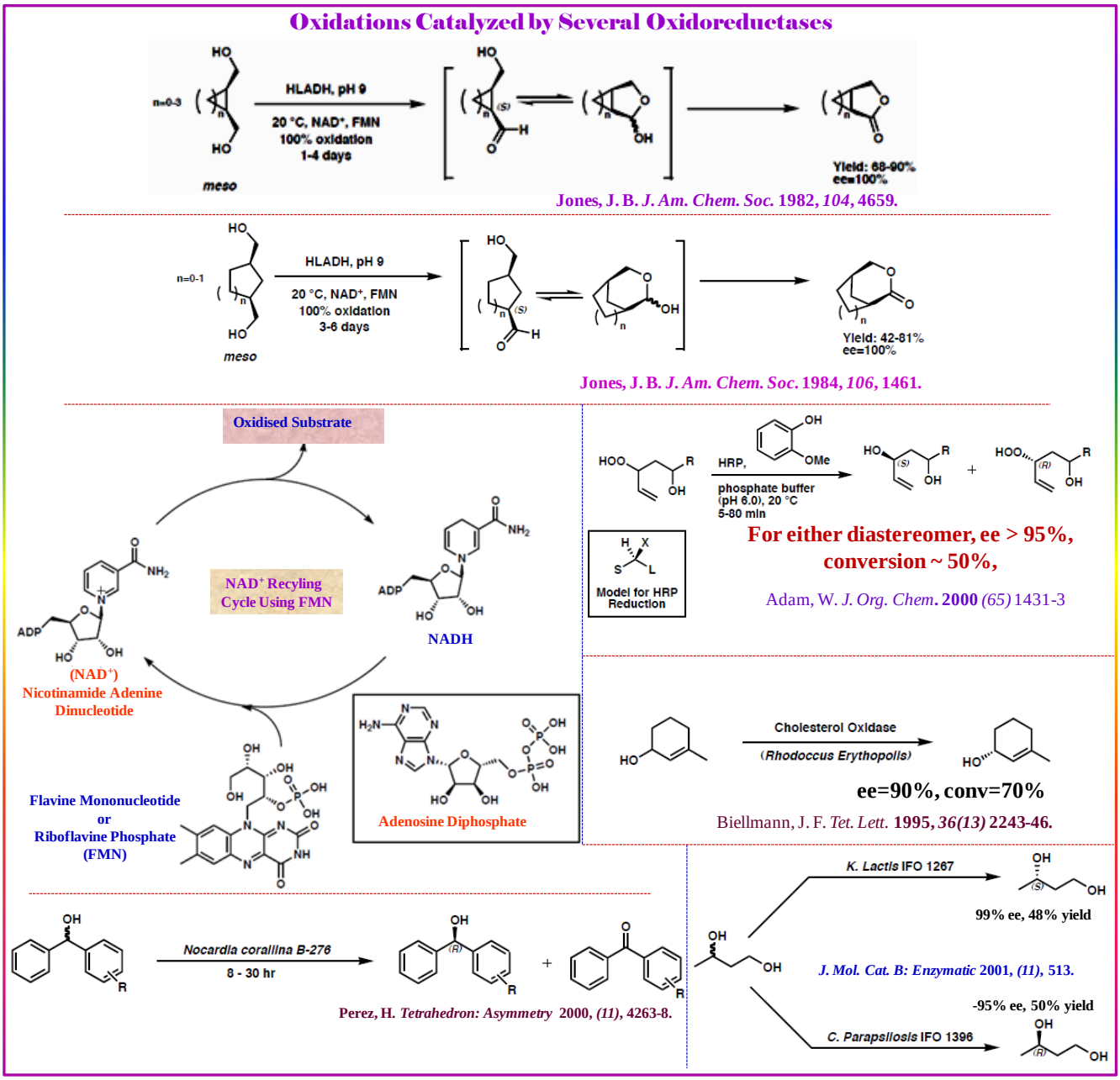
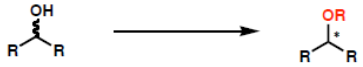


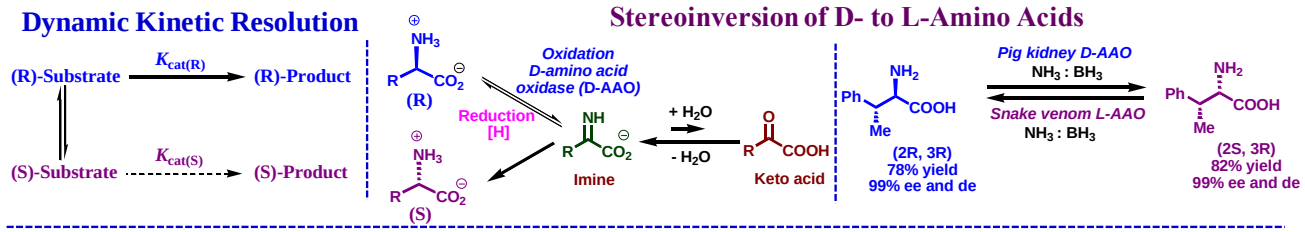
Figure 3.18: Examples of organic oxidation reactions catalyzed by oxidoreductase enzymes.

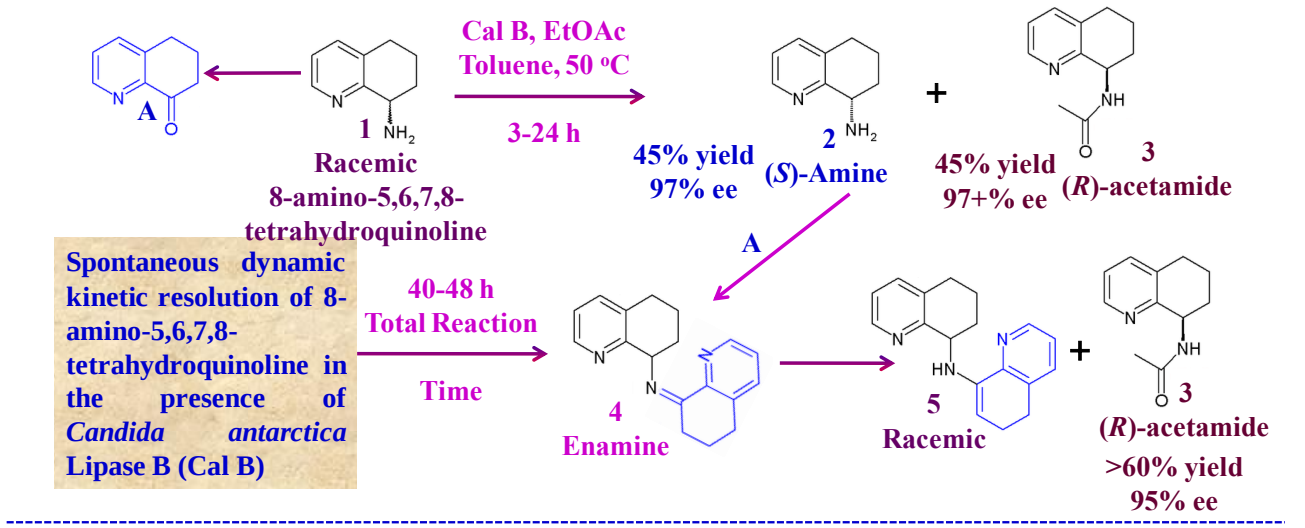
Enzymatic Dynamic Kinetic Resolutions

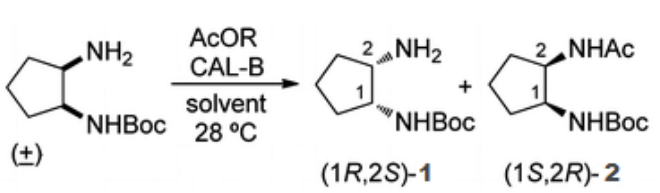
- Isolating 100% enantiopure products from racemic starting materials in quantitative yields is possible:



- by cyclic enantioselective oxidation- achiral reduction pathway
- kinetic resolution coupled with an inverse enantioselective process (e.g. reduction, Mitsunobo conditions, etc.). Or by
- in-situ racemization coupled with enantioselective transformation (e.g. enzymatic acylation followed by hydrolysis)







Francisca Rebolledo *et al. Org. Lett.* 2010, *12* (16), 3602–3605

entry	AcOR	(1*R*,2*S*)-**1** ee (%) (yield, %)	(1*S*,2*R*)-**2** ee (%)[a] (yield, %)[b]
1	AcOEt	42 (51)	94 (30)
2	$AcOCH(Ph)CH_3$	40 (62)	98 (27)
3	$AcOCH(Ph)CH_3$	97 (41)	98 (45)

Figure 3.19: Examples of organic dynamic kinetic resolution catalyzed by various enzymes.

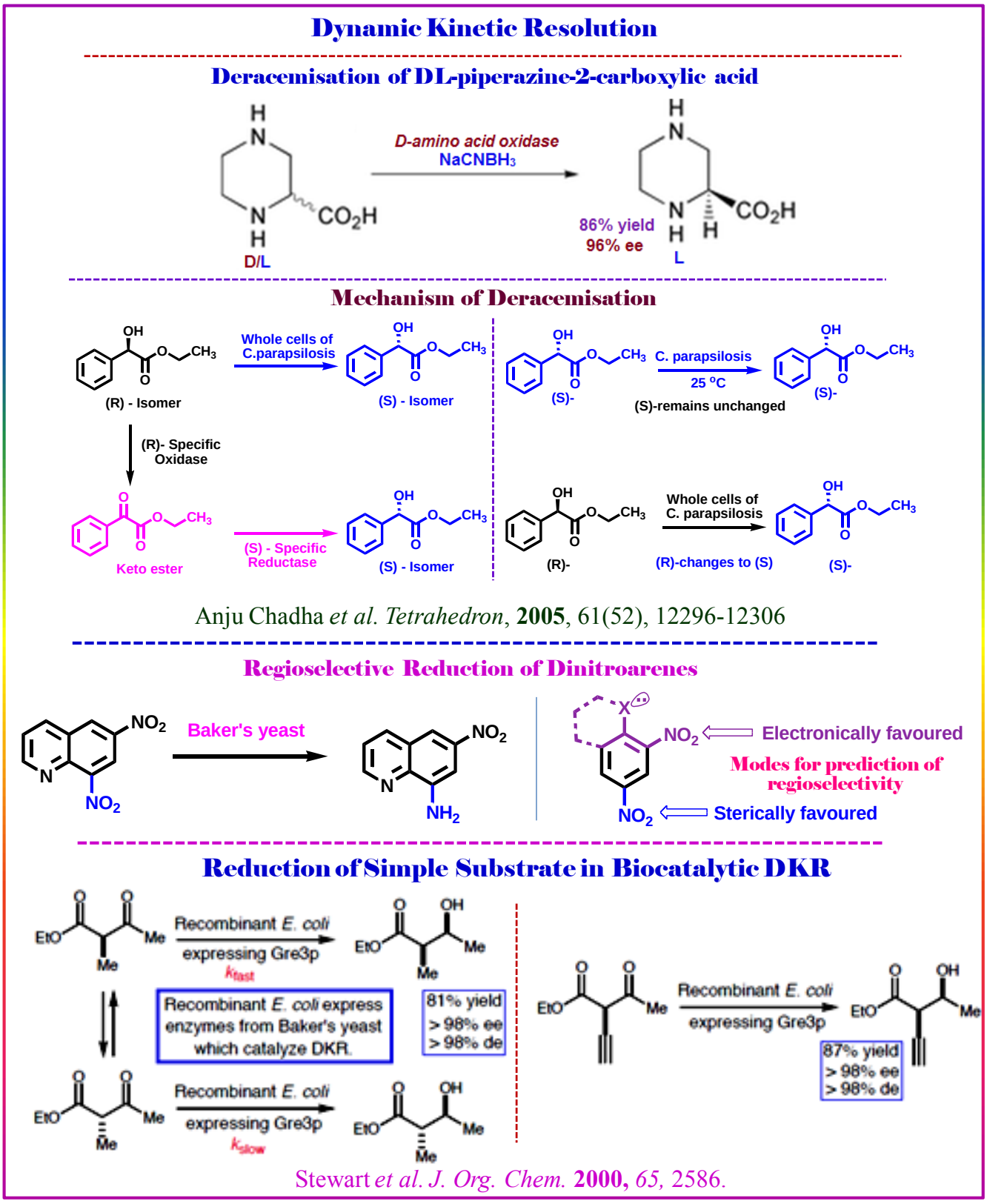


Figure 3.20: Examples of organic dynamic kinetic resolution catalyzed by various enzymes.

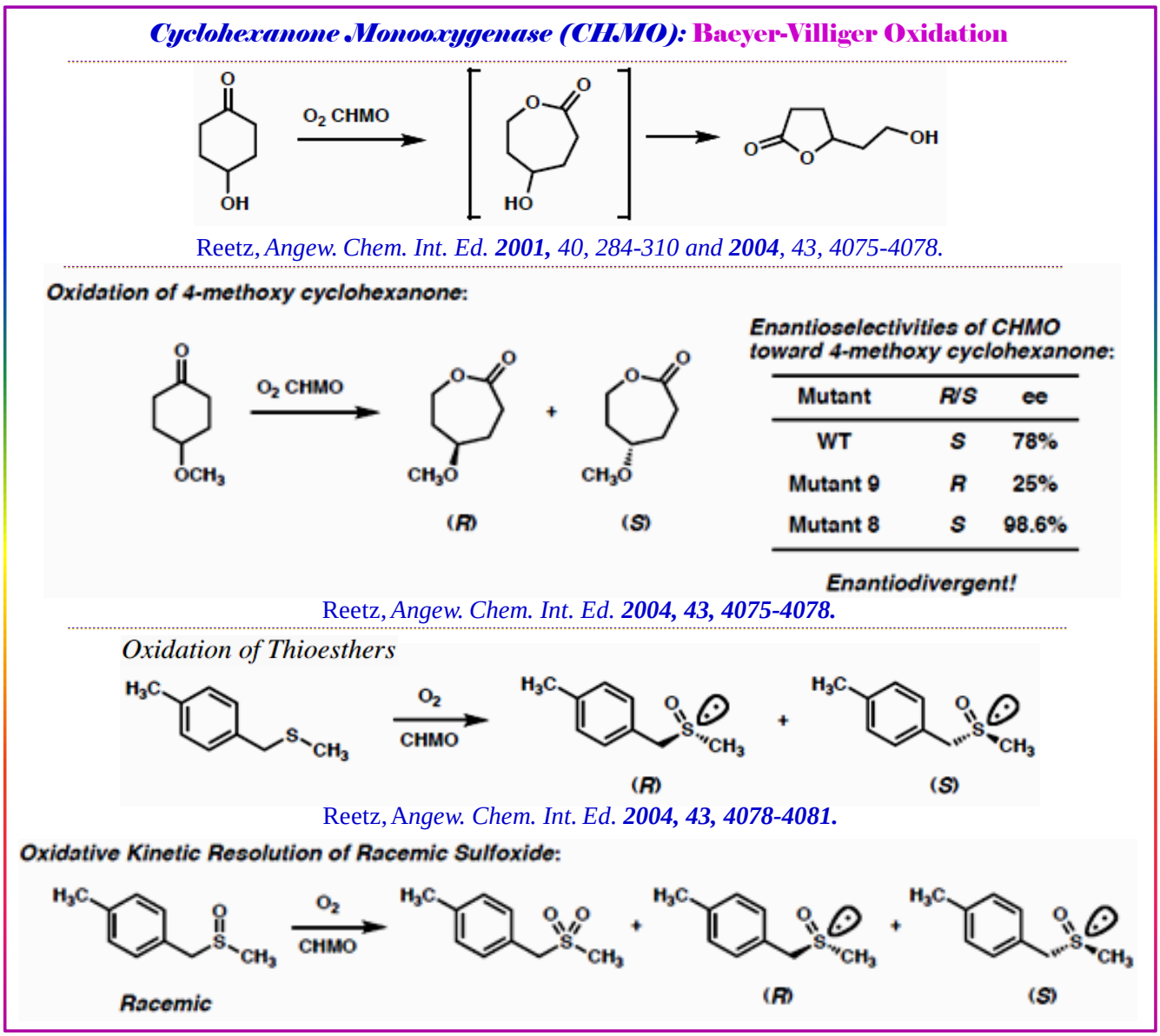


Figure 3.21: Examples of Bayer-Villiger oxidation and other oxidation reaction and oxidative dynamic kinetic resolution catalyzed by *Cyclohexanone monooxygenase (CHMO)* enzymes.

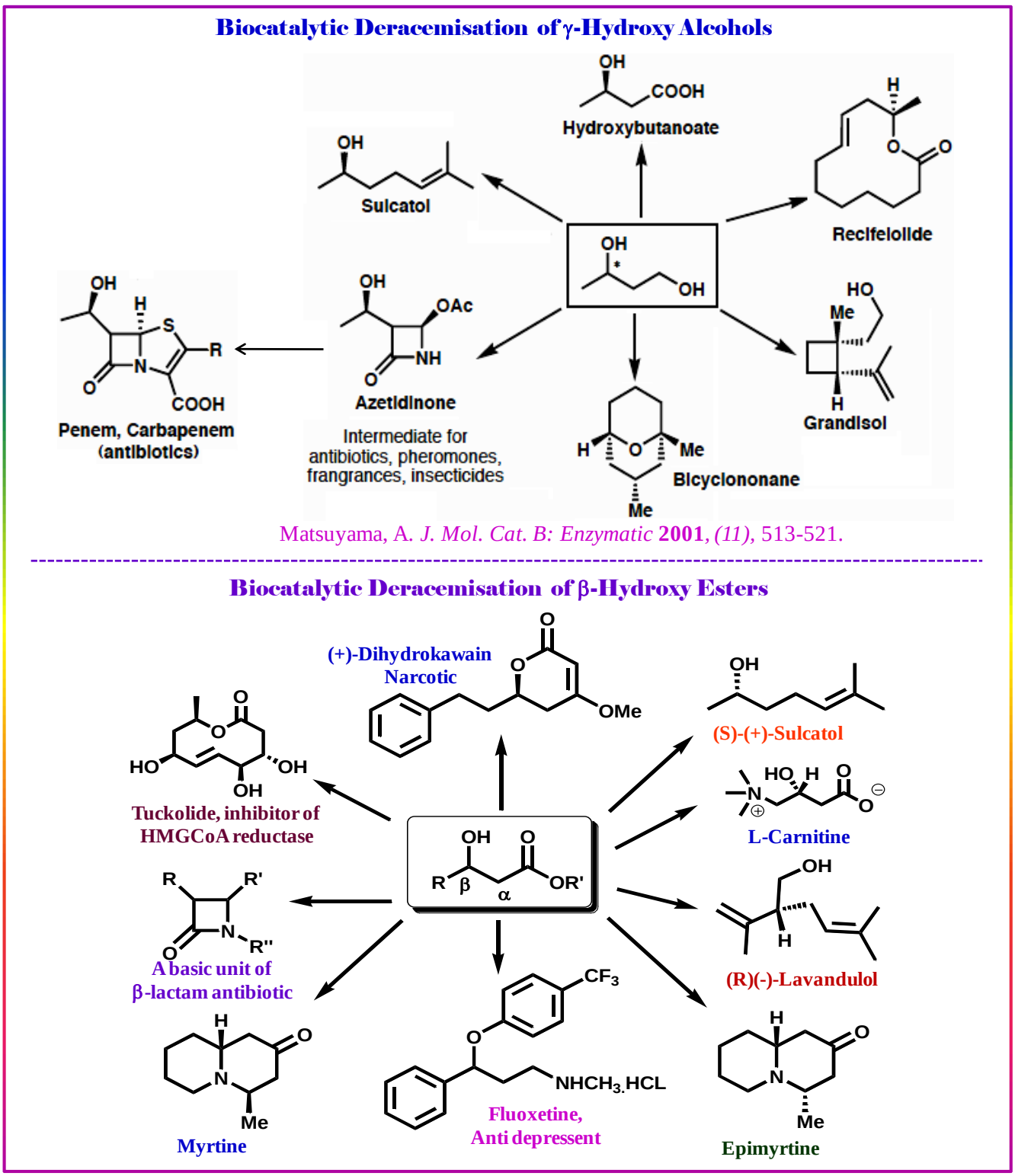


Figure 3.22: Examples of Biocatalytic deracemisation reactions.

3.11.2.2. Hydrolases (esterase, lipase): Hydrolytic reactions→Catalyze bond cleavage by the introduction of water; Hydrolysis-formation of esters, amides, lactones, epoxides, nitriles, anhydrides:

Hydrolases:

- hydrolytic cleavage of C-O, C-N, C-C and some other bonds
- favourite class of enzymes for organic chemistry
- no cofactor needed
- large number of readily available enzymes
- reversible reaction (e.g., amide- or ester-*synthesis*)
- relative high stability under non-natural conditions
- often wide substrate range

Lipases:

- Among the biocatalysts in organic synthesis, lipases are the most frequently used. In particular, this class of enzyme is able to perform enantioselective hydrolytic reactions **and** catalyzes the formation of a wide range of ester and amide bonds.
- natural function - hydrolysis or re-esterification of triglycerides (oils and fats)
- Laundry detergents, modification of natural oils and fats etc.
- offers often excellent stereoselectivity - synthesis of optically pure compounds
- Lipases are highly active in organic solvents
- the most frequently used enzymes in organic synthesis
  - Resolution of alcohols
  - Synthesis of versatile building blocks in organic synthesis
  - kinetic resolution by hydrolysis
  - kinetic resolution by acylation - activated acyl donors or enol esters (e.g., vinyl or isopropenyl acetate) reaction irreversible as alcohol generated undergo keto-enol tautomerisation

Esterases:

- split esters into an acid and an alcohol
- only few applications for synthesis of optically pure compounds
- moderate enantioselectivity and limited commercial availability

Peptidases, Amidases and Acylases:

- formation and hydrolysis of amide links
- thermodynamically controlled synthesis - shift equilibrium towards synthesis by use of organic solvents, increase concentration of starting material or removing product.
- kinetically controlled synthesis - activation of carboxyl component usually by ester or amide

- Peptide synthesis→coupling of two amino acids
- advantages of enzyme-catalyzed peptide synthesis: mild conditions , high region and enantioselectivity, no racemisation
- large scale production of Aspartame - low-calorie sweetner (DSM, NutraSweet)
- production of Kyotorphin - analgesic dipeptide (Tyr-Arg)
- synthesis of β-lactam peptide antibiotics (penicillines and cephalosporines)

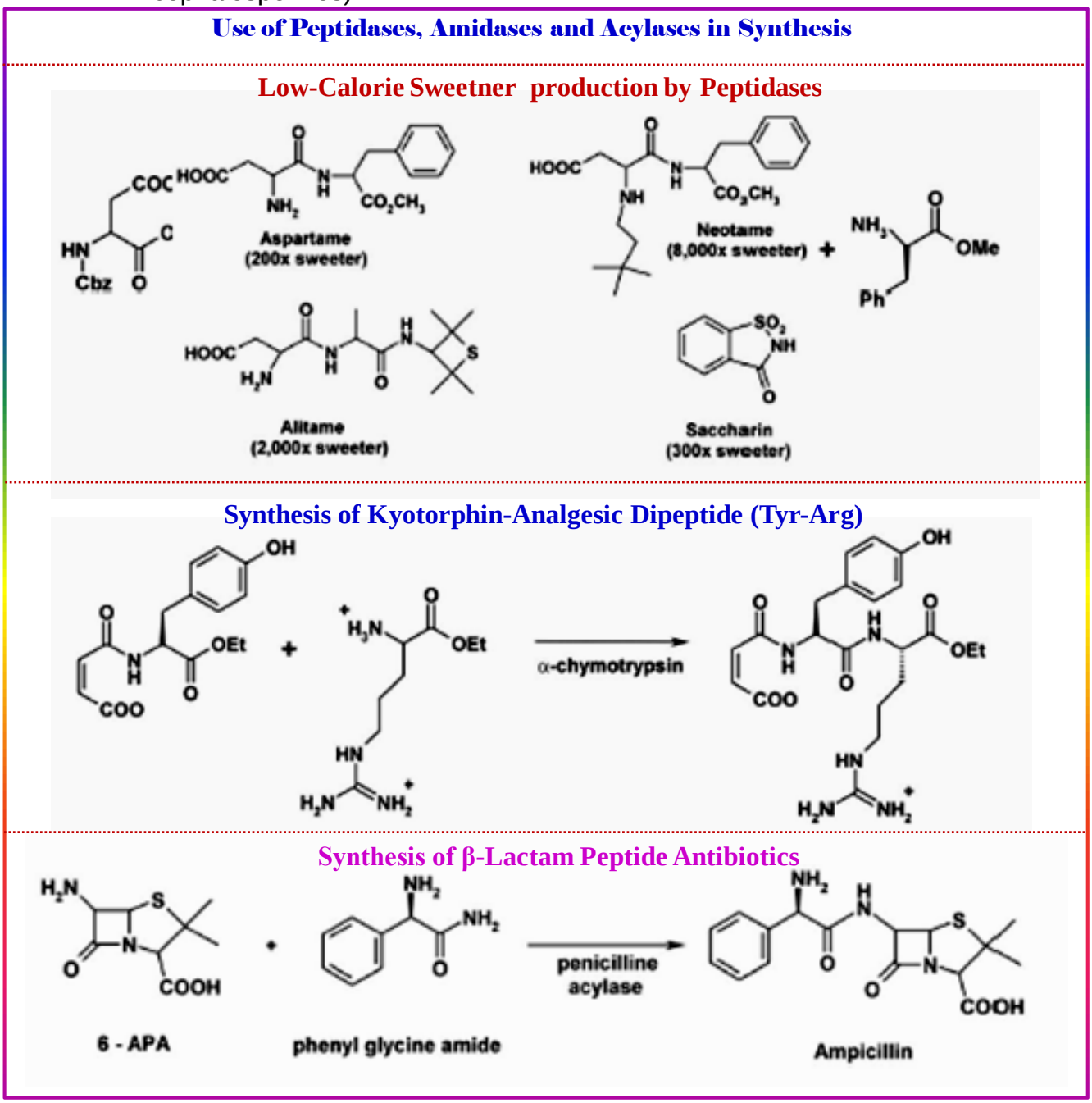


Figure 3.23: Examples of peptidases, amidases and acylases in organic reactions.

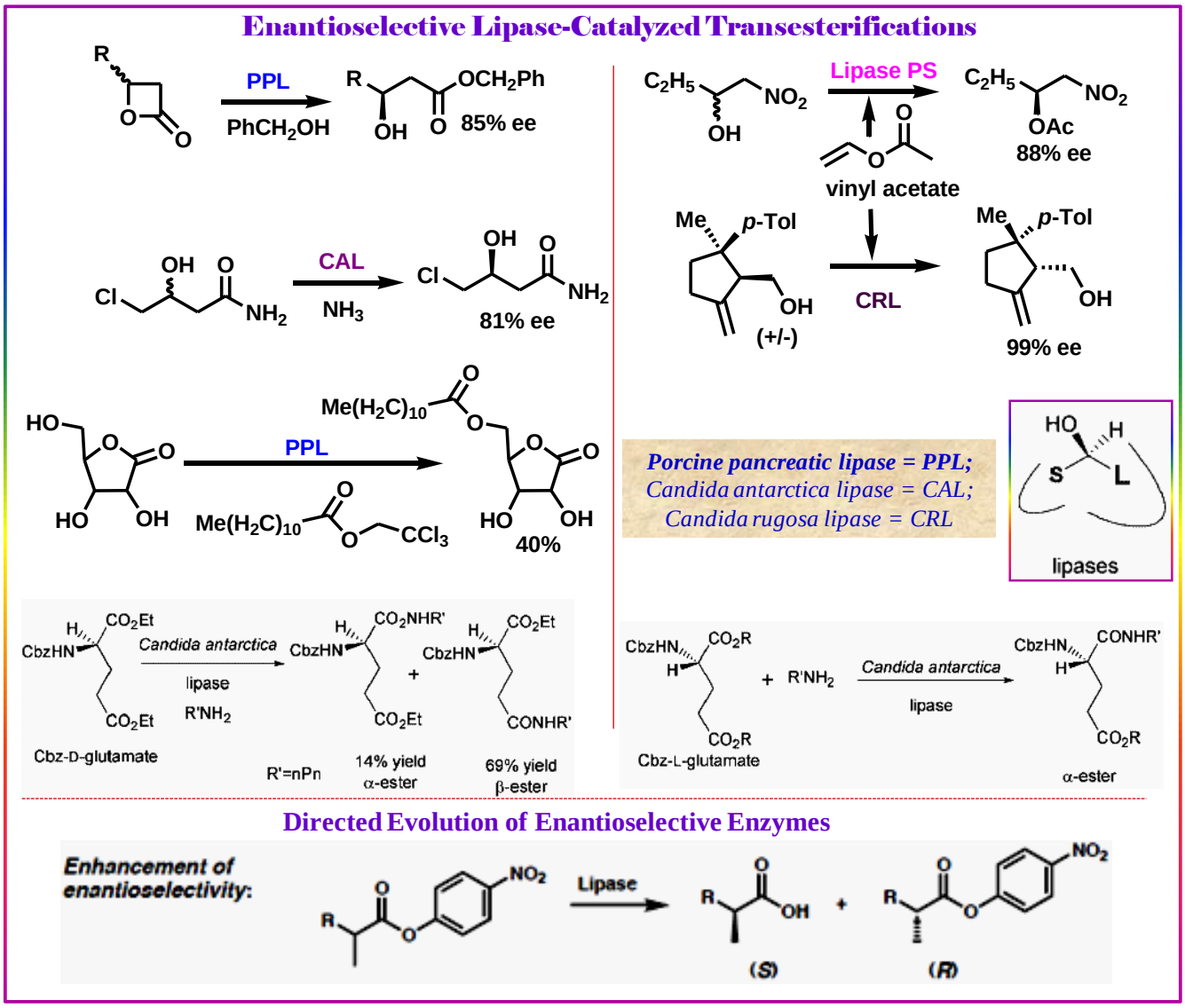


Figure 3.24: Examples of lipase catalyzed transesterification reaction.

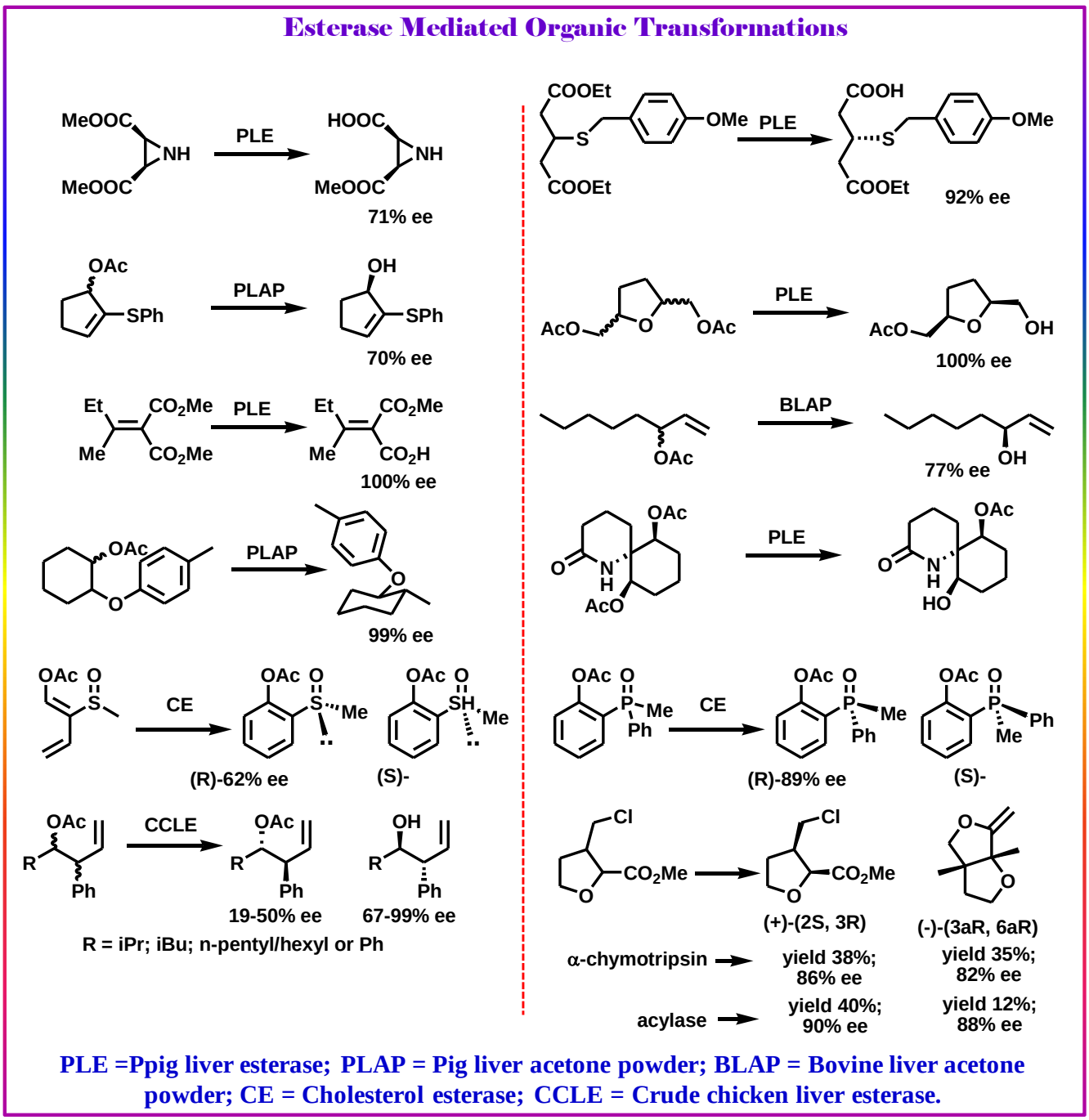


Figure 3.25: Examples of esterase catalyzed organic transformations.

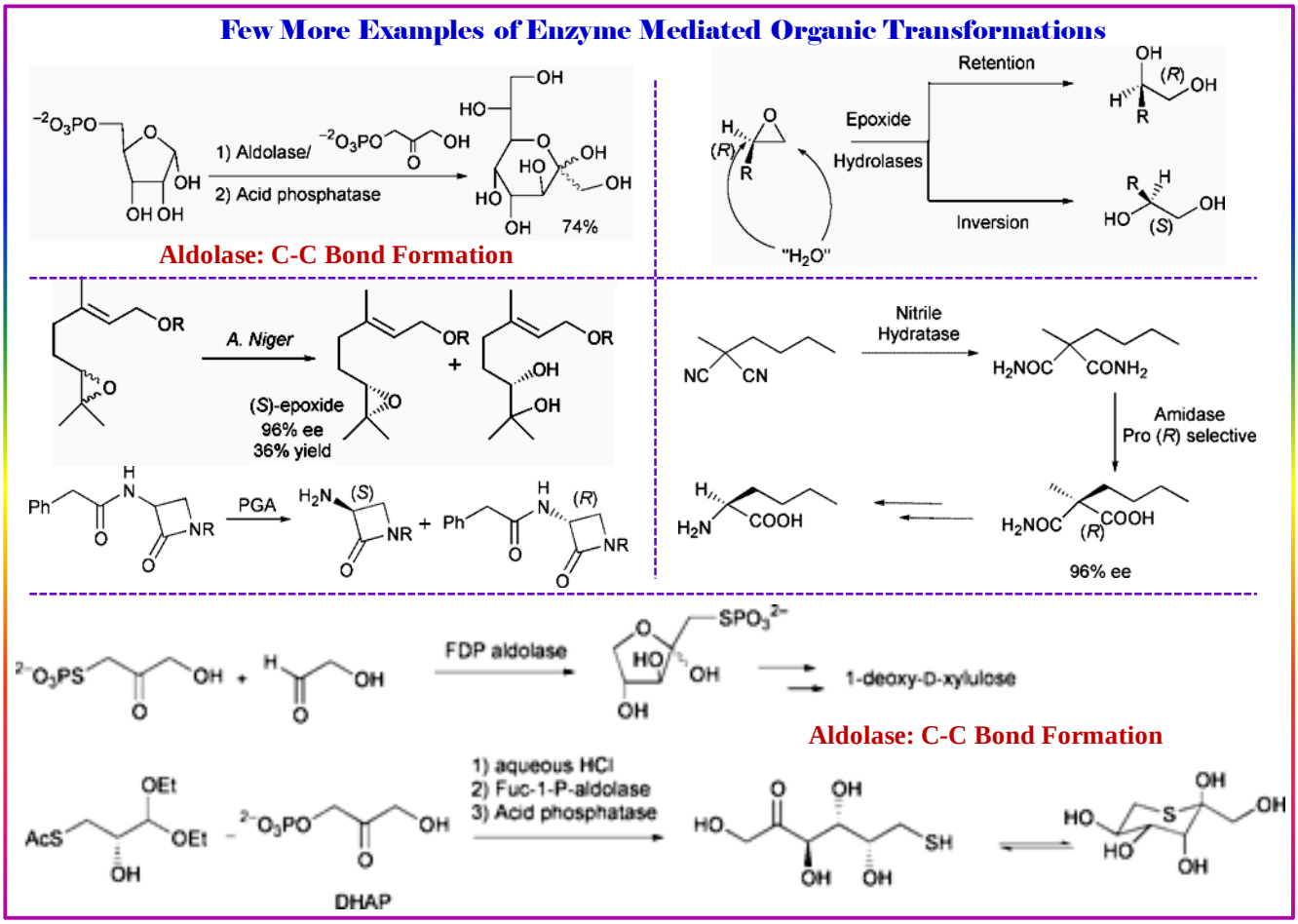


Figure 3.26: Examples of several organic transformations catalyzed by enzymes.

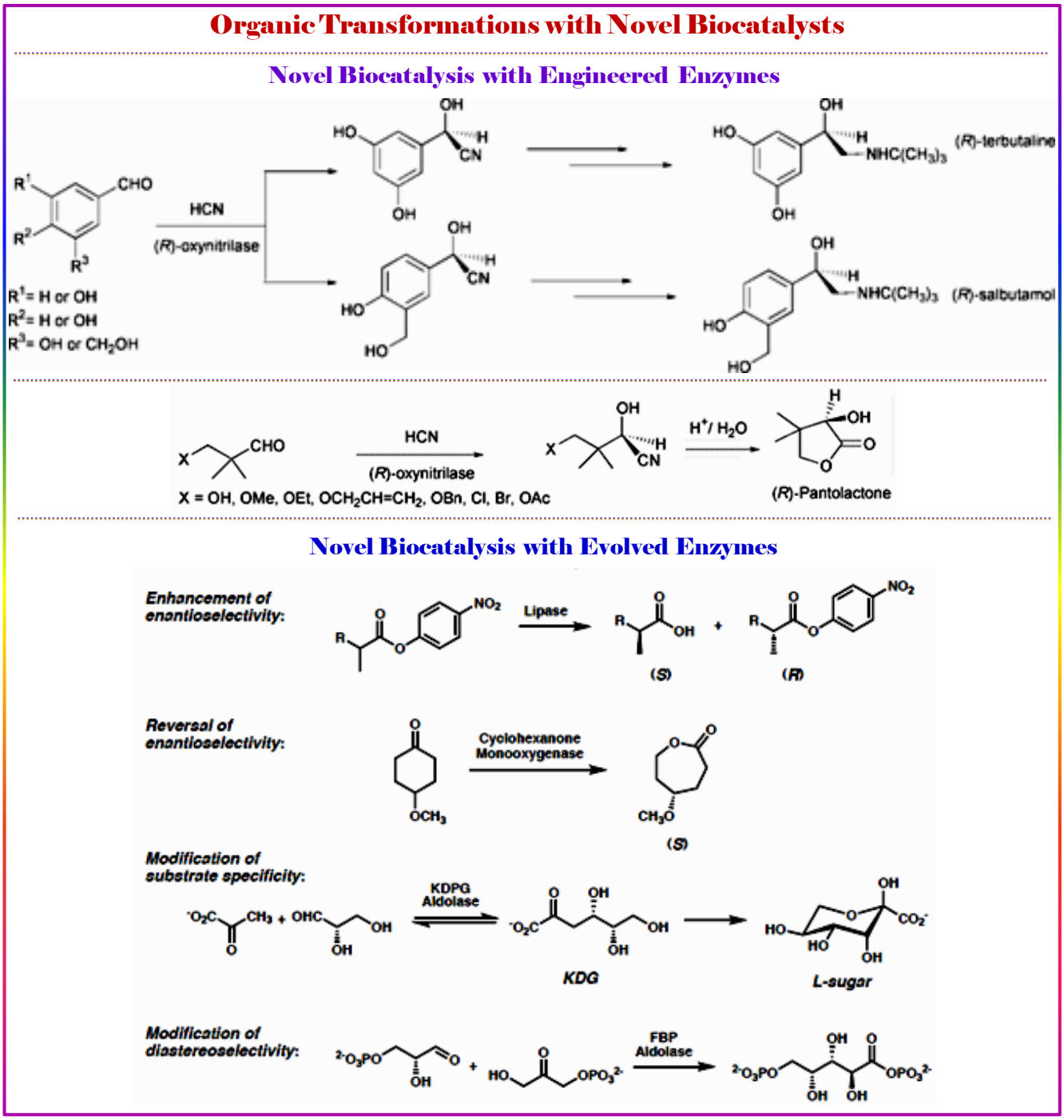
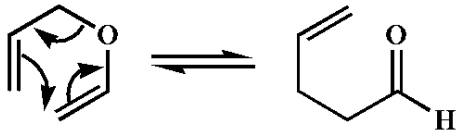


Figure 3.27: Examples of several organic transformations catalyzed by engineered enzymes.

3.11.2.3. Rearrangement Reactions Catalyzed by Enzyme:

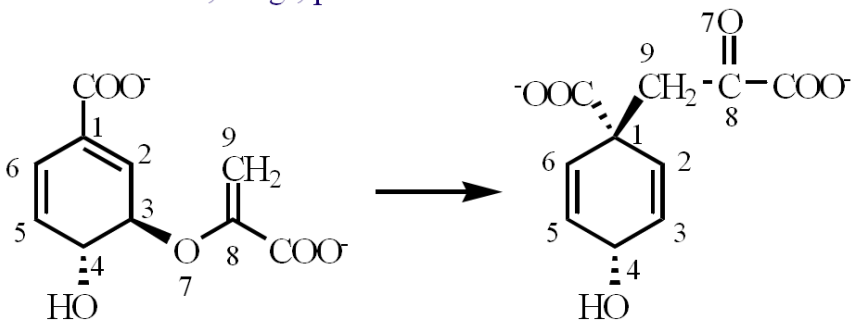
Enzyme-Catalyzed Reactions: Rearrangements

- Pericyclic Reactions:- These are concerted reactions in which bonding changes occur via reorganization of electrons within a loop of interacting orbitals. As for example,
- Sigmatropic Rearrangements: [3,3] sigmatropic rearrangement: The most important rearrangement in this class is the Claisen rearrangement which is in general form can be presented as below:



Chorismate Mutase-catalyzed Claisen rearrangement: Conversion of Chorismate to Prephenate

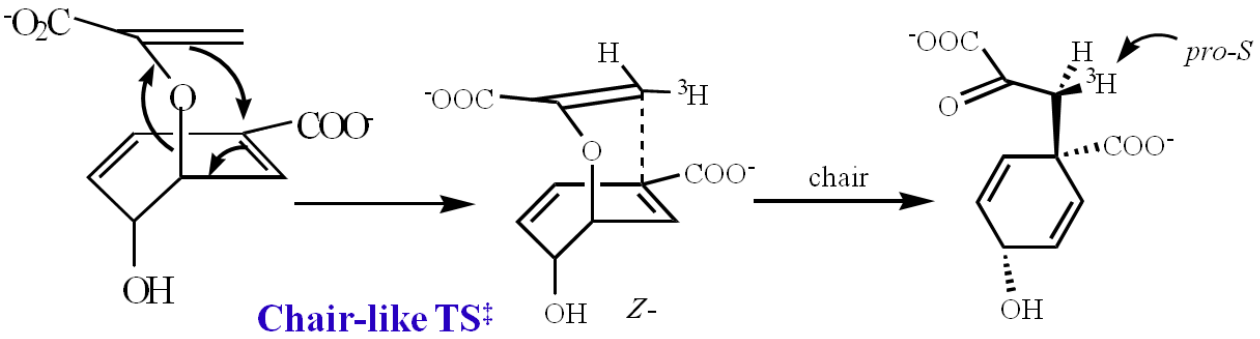
- Conversion of Chorismate to Prephenate is a step in the biosynthesis of Tyrosine and Phenylalanine in bacteria, fungi, plants.



Chorismate Prephenate

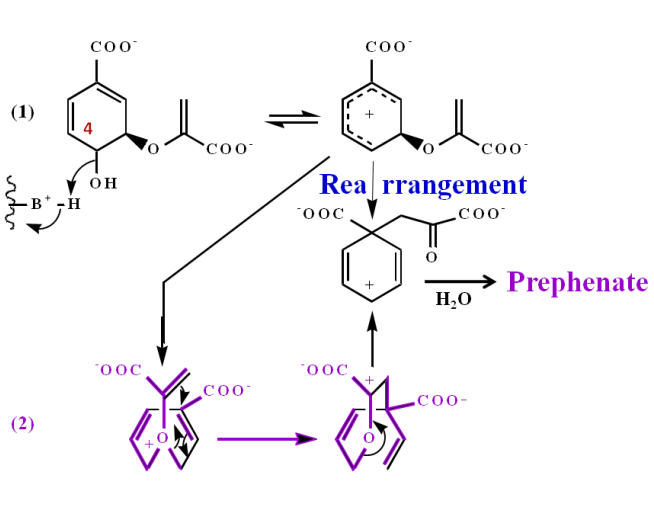
Mechanism of Claisen Rearrangement

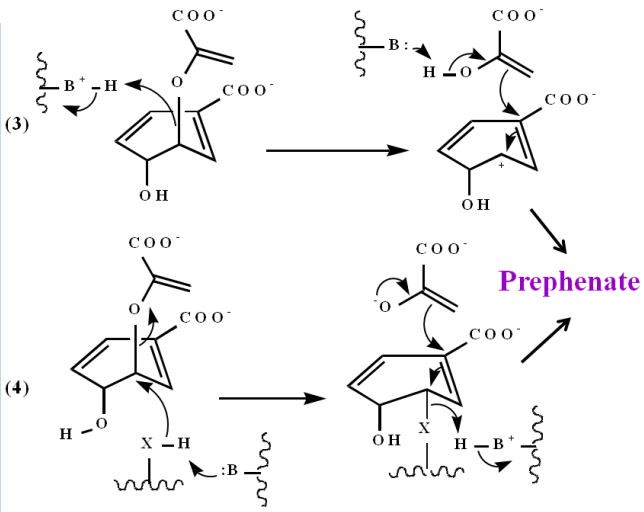
- Required conformer for Claisen rearrangement which is observed (10-40%) in solution from NMR spectrum is **Chair-like TS.**

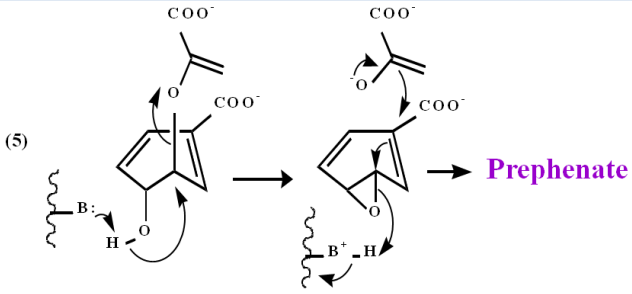


- It is evident that the Chorismate mutase proceeds via chair-like transition states, during reaction with (*Z*)-[9-^{3}H] chorismate.

Hypothetical Stepwise Mechanisms for the Reaction Catalyzed by Chorismate Mutase

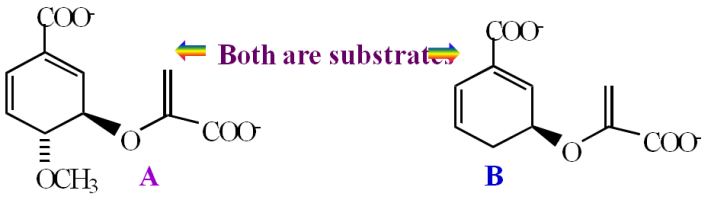






- 2° inverse isotope effect on C-4 ($sp^2 \rightarrow sp^3$); therefore mechanism 1-3 ($sp^3 \rightarrow sp^2$) are not operating.
- **Mechanism 1-3 and 5:** General Acid-Base Catalysis
- **Mechanism 4:** Nucleophilic Catalysis. None of them are operating.
- So, only possiblility is via chair like transition state geometry.

- Considering followings two substrates (A and B) as the substrates for chorismate mutase, following conclusion can be drawn.
- 16 mutants were made to show neither general acid-base catalysis (mechanisms 1-3, 5) nor nucleophilic catalysis (mechanism 4) is important in the Reaction Catalyzed by Chorismate Mutase.



Mechanism 5 Excluded **Mechanisms 1, 2, 5 Excluded**

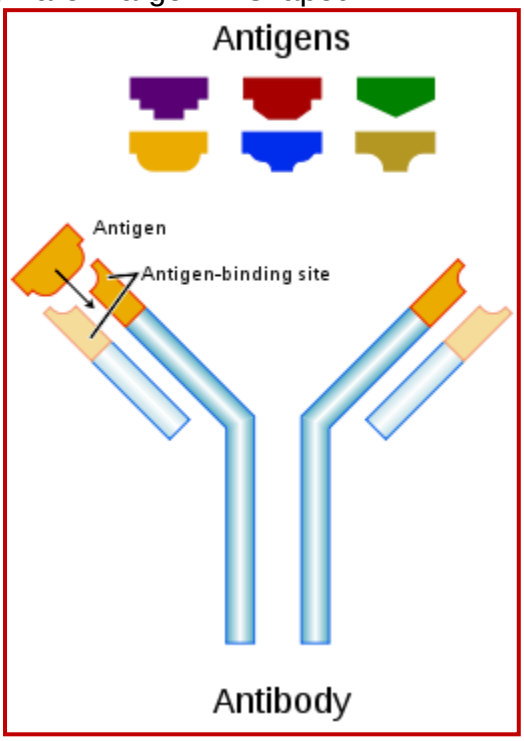
Conclusion:

- It is a Pericyclic reaction and the function of the enzyme is to stabilize the chair like transition state geometry.

3.12. Antibody Catalyzed Organic Reaction

Combinatorial process in nature drives the combinatorial strategy of generating large libraries of small molecules of potential applications and is the synergy between chemistry and biology. As for example, the same combinatorial strategy is used by Human Immune Systems to generate large libraries of "Antibodies" and to select antibodies from this large library that can recognize foreign antigens with high affinity and selectivity.

Thus, A**ntibodies,** known as **immunoglobulin**, are large Y-shaped glycoproteins made of basic structural units—each with two large heavy chains and two small light chains, used by the humoral immune system to identify and neutralize foreign organism like bacteria and viruses. The antibody recognizes a unique part of the foreign target, termed an antigen. Each arm of the "Y-shape" of an antibody contains a paratope-a structure analogous to a lock that is specific for one particular epitope- analogous to a key on an antigen. Thus, interaction/binding between the antibody and a foreign antigen follow the "lock-key" mechanism similar to enzyme. Using this binding mechanism, an antibody can *tag* a microbe or an infected cell for attack by other parts of the immune system, or can neutralize its target directly. For example, antibody blocks a part of a microbe that is essential for its invasion and survival. Thus, the production of antibodies is the main function of the humoral immune system to fight against infection due to foreign organism. There are five different antibody isotypes, based on heavy chain they possess, are known in mammals, which carry out different roles, and help direct the appropriate immune response for each different type of foreign object they encounter.



3.12.1. Transition-State Theory of Catalytic Antibodies:

We now know that most chemical transformations in the biological world are catalyzed by enzymes and many are by catalytic ribozymes. Enzymes are thus important biocatalysts and show their catalytic activity with profound selectivity and specificity. Therefore the idea came to the mind that if one can create designer protein catalysts that exhibit enzyme like selectivity and specificity would facilitates an understanding of how enzymes work and evolved. In addition, such designer proteins can catalyze chemical transformations on demand even when no natural enzyme does so. In 1940, Linus Pauling suggested the idea that enzyme catalysis is due to a selective recognition of the transition state. In the same year he published also the first paper on the structure of an antibody, and he discussed first the mechanism of recognition in immunocomplexes. "*In order to catalyze a reaction an enzyme must recognize*

the transition state in a selective way, i.e. it must stabilize it better than the substrate."

The concept of transition state binding and stabilisation of the same by catalytic enzymes, is an extension of the new transition state theory developed by Evans *et al.* and Eyring *et al.* to explain chemical catalysis. The basic proposition is that the rate of a reaction is related to the difference in Gibbs free energy (ΔG^0) between the ground state and the transition state of the reaction. Catalysis proceeds smoothly either by lowering the energy of the transition state i.e. by transition state stabilization or by elevating the energy of the substrate i.e. by substrate destabilization. Pauling has applied this concept to enzyme catalysis by stating that an enzyme preferentially binds to and thereby stabilizes the transition state for a reaction relative to the ground state of substrate(s). This has become a classical theory in enzymology and is widely used to explain the rate accelerations of an enzyme catalyzed reaction. The idea of creating catalytic proteins along with the transition state analogues directs the generation such designer protein catalysts popularly known as "Catalytic Antibodies" or Abzymes".

The concept that an antibody raised against a transition state analogue could catalyze a reaction was first described by Jencks in **1969 (**William p. Jencks, ***Catalysis in Chemistry and Enzymology***, **1969**, p.288**) f**ollowing the fundamental principles of Pauling: *"If complementarity between the active site and the transition state contributes significantly to enzymatic catalysis, it should be possible to synthesize an enzyme by constructing such an active site. One way to do this is to prepare an antibody to a haptenic group, called "Hapten" which resembles the transition state of a given reaction. The combining site of such antibodies should be complementary to the transition state and should cause an acceleration by forcing bound substrates to resemble the transition state."* In 1979 Köhler and Milstein described a method to obtain monoclonal hybridoma cells, producing homogeneous preparations of a single antibody. This discover opened the way also to antibody catalysis. The energy profile diagram and the kinetic equation of transition state stabilization by antibody is shown in figure 3.28.

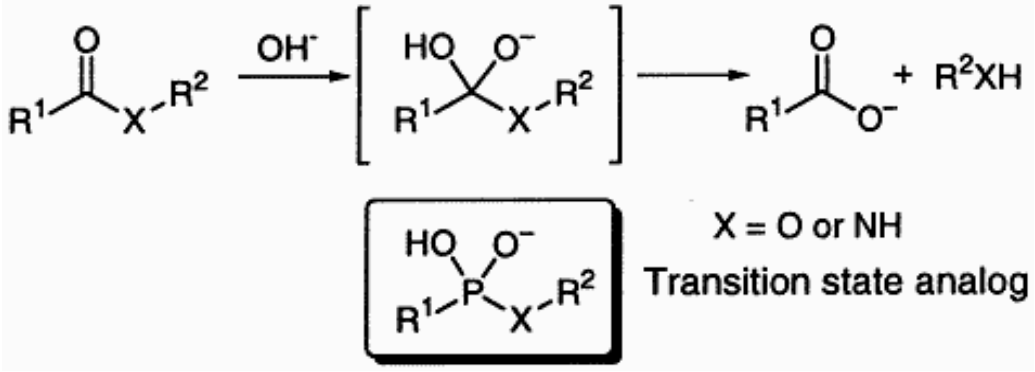
The early examples of antibody catalyzed reactions are based on the TS of ester/amide hydrolysis.

- Ester and amide hydrolysis:
    - involve the formation of a high-energy tetrahedral intermediate
    - Mimicking the transition state of this tetrahedral intermediate by using phosphonates and phosphonamidates
    - Antibodies bind specifically the transition-state analogue and stabilize it more compared to the ground-state substrate.
    - the rate enhancement is expected from the transition-state stabilization

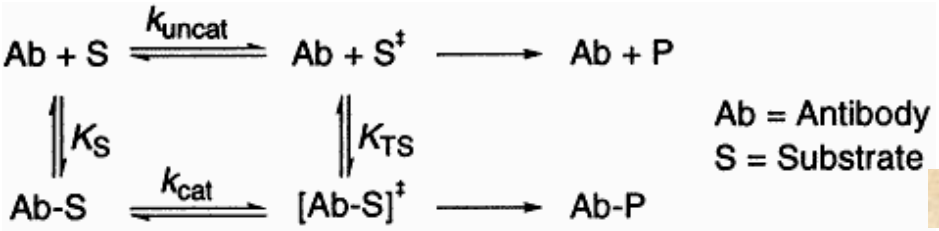
Transition-State Analogs And Catalytic Antibodies

Mechanism of Ester and Amide Hydrolysis

- A tetrahedral intermediate
- the phosphonate (X = O) or phosphonamidate (X = NH) transition-state analogue.



Substrate and Transition-State Binding for an Antibody



$k_{cat}K_{TS} = k_{uncat}K_S$

$k_{cat}/k_{uncat} = K_S/K_{TS}$

K_S = Dissociation constant for the antibody–substrate complex; K_{TS} = Dissociation constant for the antibody–transition-state complex; k_{cat} = Rate constant for the reaction of the antibody–substrate complex; k_{uncat} = Rate constant for the reaction without antibody.

Energy Profile for the Antibody-Catalyzed Reaction

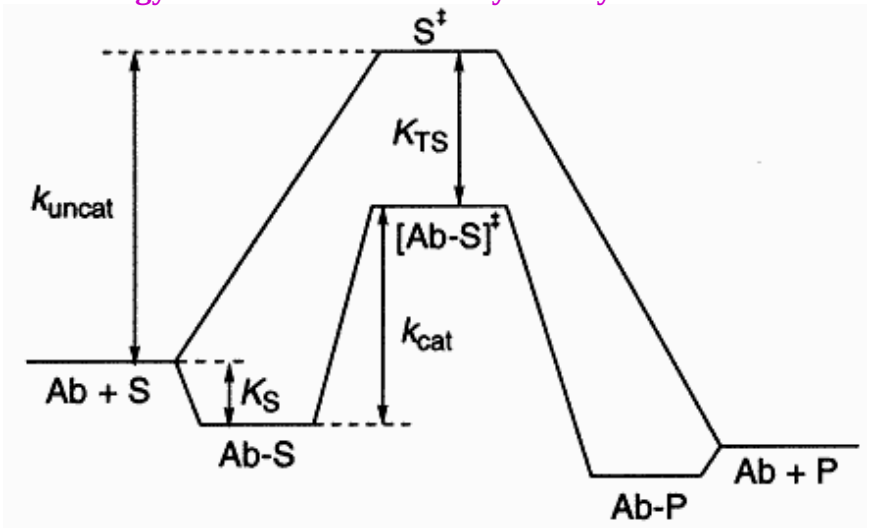


Figure 3.28: Transition state theory of Catalytic Antibody.

3.12.2. Generation of Monoclonal Antibody (mAb)

The first monoclonal catalytic antibodies were reported by the Lerner group and Schultz's group in 1986. Lerner group have generated catalytic antibodies by immunization with a phosphonate transition-state analogue designed for ester hydrolysis while Schultz's group showed that the phosphate diester transition-state analogue binding antibody is a catalyst. Since then, antibody catalysts have attracted attention of several research groups for carrying out many types of reactions with catalytic diversity and specificity. Therefore, now, catalytic antibodies have been obtained not only with transition-state analogues but also with other designed compounds.

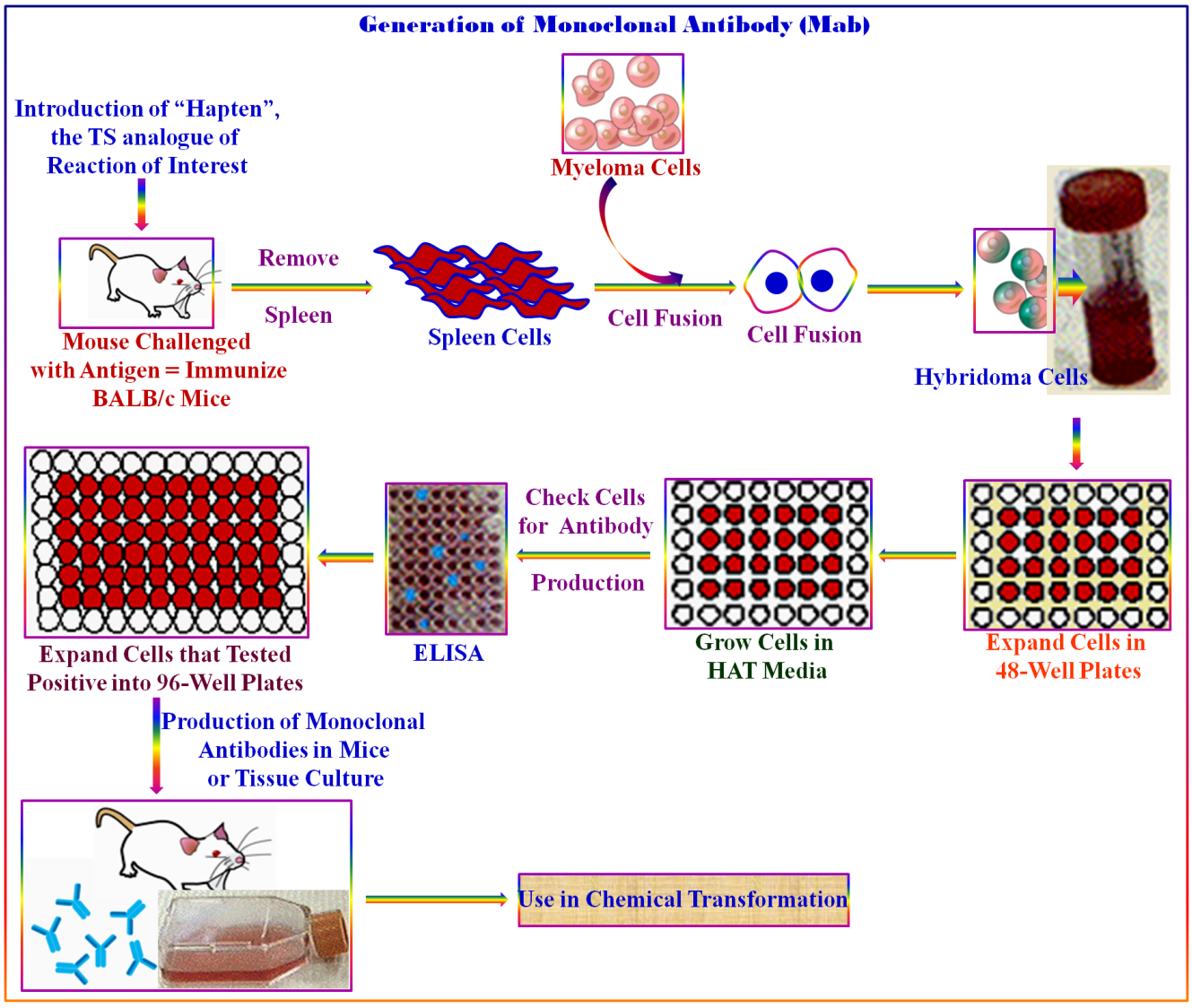


Figure 3.29: Method of generation of monoclonal Antibody (mAb).

3.12.3. Approaches to Hapten Design

Based on the principal elements Haptens can be designed by the following five ways out of these first four will be discussed here.

1. Transition state analogs.
2. Bait and switch.
3. Reactive Immunisation/Supplementation of chemical functionality.
4. Cofactor Approach
5. Entropy traps.
6. Desolvation.

3.12.3.1. Catalytic Antibodies Generated Against Transition-State Analogs (TSAs):

Haptens are the mimic of stable transition state (TS)/a high-energy intermediate of a reaction of interest. Such TS analogues induce the generation of catalytic antibodies and are the foundation of abzyme research. Therefore knowledge of mechanistic pathway of any reaction can give idea of its TS and thus it is possible to synthesis the hapten easily.

3.12.3.1.1. Antibody-catalyzed acyl transfer reactions

Ester and amide hydrolysis are known to involve high-energy tetrahedral intermediates. This intermediate decomposes into the corresponding carboxylic acid and alcohol or amine, respectively. This high-energy intermediate and corresponding transition state can be mimicked by phosphonates, phosphonamidates, arsonates, and sulfonates, all of which contain the key tetrahedral structural motif and are often employed in the design of Transition State Analogue (TSA) haptens for immunization. Catalytic antibodies are thus designer proteases and esterases.

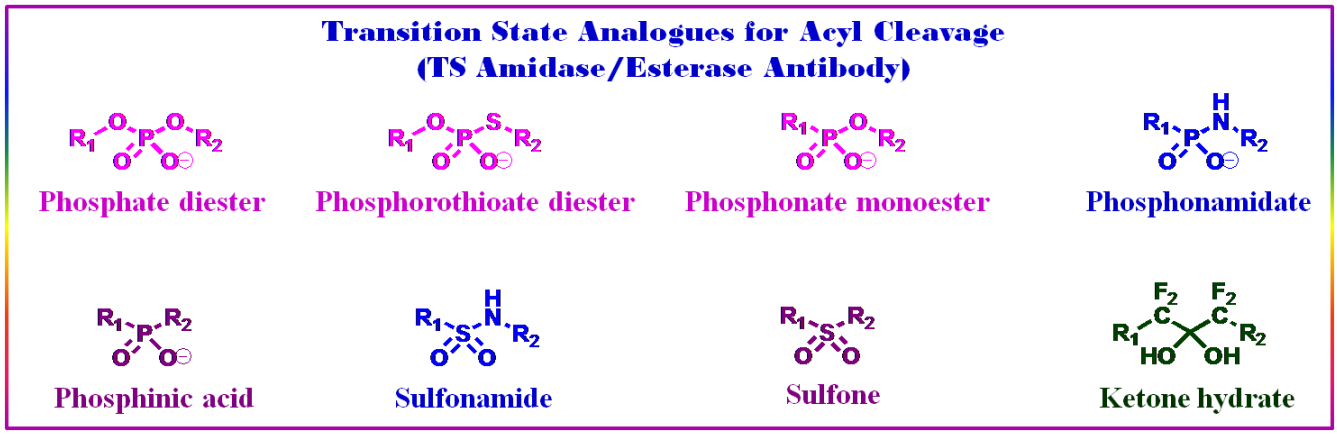


Figure 3.30: Various TSAs for acyl-cleavage by catalyzed by monoclonal Antibody (mAb).

In the tetrahedral intermediates used to generate TS analogue, phosphorus (V) systems is considered because of strong polarisation of P-O bond (P^{+}-O^{-}). The range has included many of the possible species containing an ionized P–OH group. Most important feature of such systems is that the P–O bond is intermediate in length (1.521 Å) between the C–O bond of an anionic tetrahedral intermediate (0.2–0.3 Å shorter) and for the C · · ·O breaking bond in the transition state (0.6 Å longer). Most of these antibodies fulfill the relationship $k_{cat}/k_{uncat} = K_S/K_{TSA}$. Amide hydrolysis is a difficult task due to the fact that an amine is in general a very poor leaving group. In the design of amidase antibodies, phosphinates, and phosphonamidates TS are preferred.

One such example is antibody 43C9, which not only catalyzes the hydrolysis of aromatic amides and esters, but also shows exceptional rate acceleration. It is induced with a tetrahedral transition state mimic, phosphonamidate. The reaction and the mechanism of amide/ester hydrolysis are shown in **Figure 3.31** below.

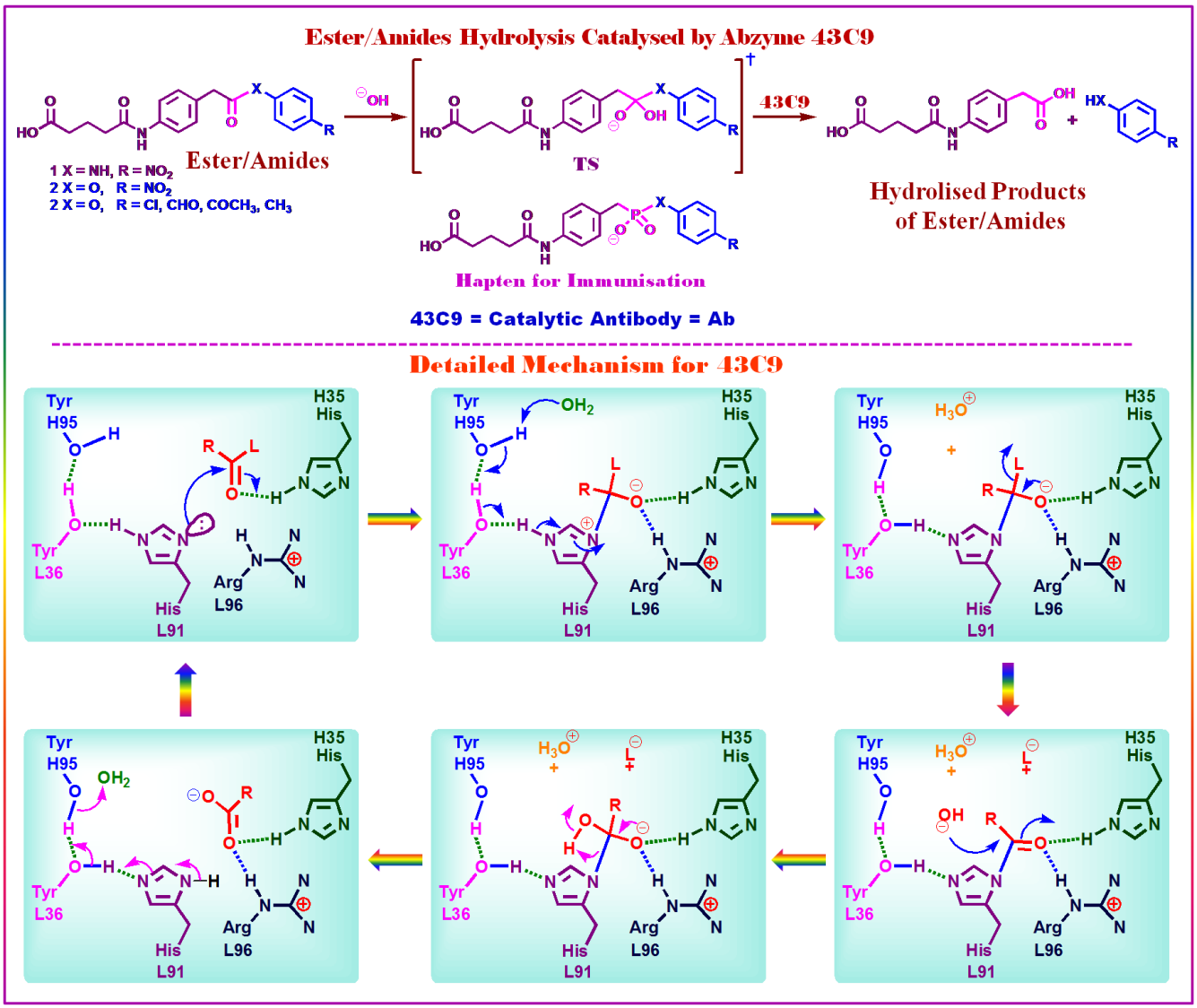


Figure 3.31: Detailed mechanism for 43C9 catalyzed ester/amide hydrolysis.

The early experiments involved the generation of esterolytic/amidolytic antibodies involving phosphonate/phosphate/phosphonamidate transition state analogues. Since then, antibodies have been developed to catalyze a wide range of chemical and biological reactions which involve acyl transfer to water oxidation. Thus, antibody shows activity similar or even exceeding those of enzymes. In a number of cases such as catalysts for pericyclic, acyl transfer, metallation, and adol reactions, antibodies have been found to have rates and mechanisms comparable to those of known enzymes.

Below are few more examples of antibody catalyzed different types of reactions involving Transition state analogue approach of eliciting antibodies.

3.12.3.1.2. Antibody-catalyzed cationic cyclization

Antibody catalyzed tandem cationic cyclization of a polyene substrate was reported. One such example is antibody HA519A4 (**Figure 3.32A**) the co-crystallized of the Fab fragment of which with its eliciting hapten 1, a TSA, showed that the hapten is deeply buried within a hydrophobic pocket. The antibody combining site provides a highly complementary fit as well as multiple aromatic residues. Therefore, upon binding the polyene, the active site of the antibody forces it into the productive chair-chair conformation.

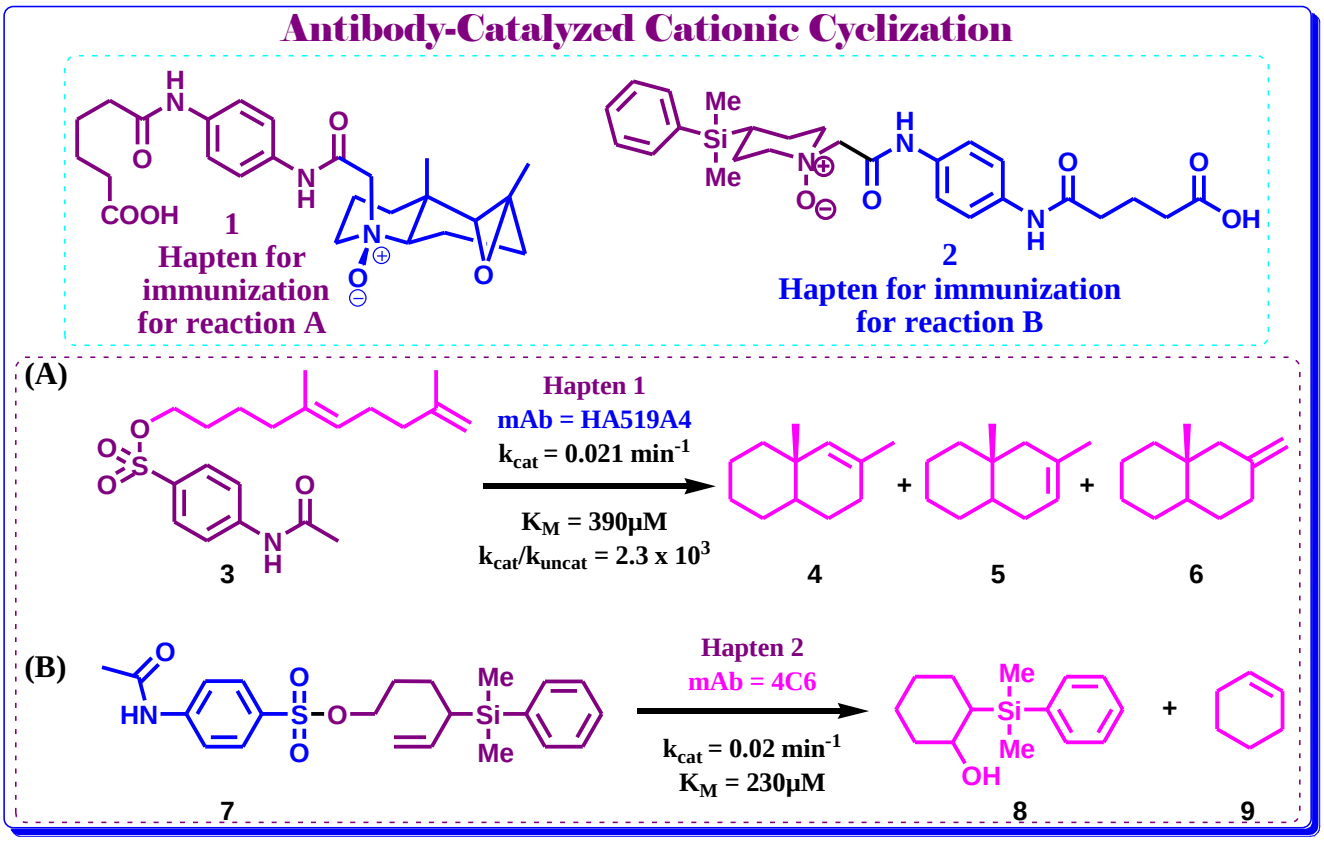


Figure 3.32: Cataionic cyclization reaction catalyzed by mAb HA519A4 and 4C6 antibody and the structures of the TSA haptens.

Antibody 4C6 elicited against hapten 2, is another example that catalyzes another cationic cyclization reaction (**Figure 3.32 B**). The active site with aromatic residues shields the high-energy intermediate from solvent and stabilizes the carbocation intermediates through cation–π interactions, thus enabling the reaction.

3.12.3.1.3. Antibody-catalyzed disfavoured ring closure

Rerouting reaction pathways is the dramatic feature of antibody catalysts. Thus, disfavoured chemical transformations instead of favored low-energy chemical processes can be achieved with the help of antibody-catalyst. One such exciting example is the antibody-catalyzed disfavoured 6-*endo*-intramolecular cyclization reaction of trans-epoxyalcohol which (**Figure 3.33**) is disfavoured process due to the significant stereoelectronic constraints predicted by Baldwin's rules. The uncatalyzed cyclization of trans-epoxyalcohol proceeds *via* the 6-exo pathway, affording tetrahydrofuran following the Baldwin's rules. Hapten 10a was designed to mimic the stereoelectronic features of the disfavoured 6-*endo*-transition state, where the *N*-oxide functional moiety mimics the electronic polarization of the epoxide in the transition state. The piperidinium ring provides the required pyran chair conformation of the disfavoured product. Antibody 29D9 was obtained, which was able to redirect the reaction process towards yielding tetrahydropyran as the only product, a predicted product of disfavoured ring closer pathway.

The Hapten, *N*-methyl ammonium 10b was used in the immunization to elicit antibody 5C8 which catalyzed disfavoured *endo*-ring opening of the substrate with high regio- and enantio-selectivity. The active site of the antibody contains a putative catalytic diad, consisting of AspH95 and HisL89 that perform general acid/base catalysis to perform disfavoured ring closer reaction.

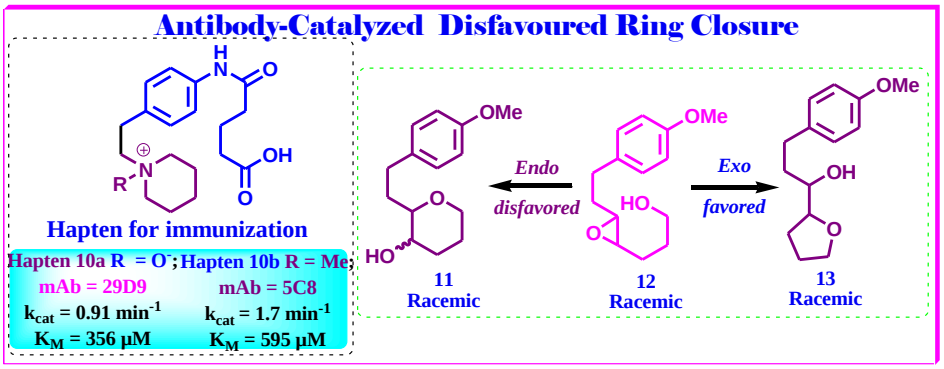


Figure 3.33: Antibody catalyzed disfavoured ring closure reaction catalyzed by mAb 29D9 and 5C8 and the structures of the TSA haptens.

3.12.3.1.4. Antibody-catalyzed Diels–Alder reaction

The Diels–Alder reaction is of particular interest for chemists because it is a rare reaction in nature, and it proceeds via an entropically disfavoured, highly organized transition state. To date, a number of antibody Diels–Alderases have been reported.

Thus, antibody 1E9 was elicited against the *endo*-hexachloronorbornene derivative 14, a stable analog of the high-energy transition state for the cycloaddition between tetrachlorothiophene dioxide and *N*-ethylmaleimide (**Figure 3.34A**). From the X-ray crystallographic data, it was revealed that the antibody binding pocket is preorganized to provide significant shape complementarity with the hapten through Van der Waals contacts, π-stacking with the maleimide functional moiety, and a hydrogen bond with AsnH35.

Kim et al. surveyed Diels–Alder reactions catalyzed by noncovalent binding to synthetic, protein, and nucleic acid hosts and reported that the antibody 1E9 is the most effective catalyst of the noncovalent catalyst systems studied.

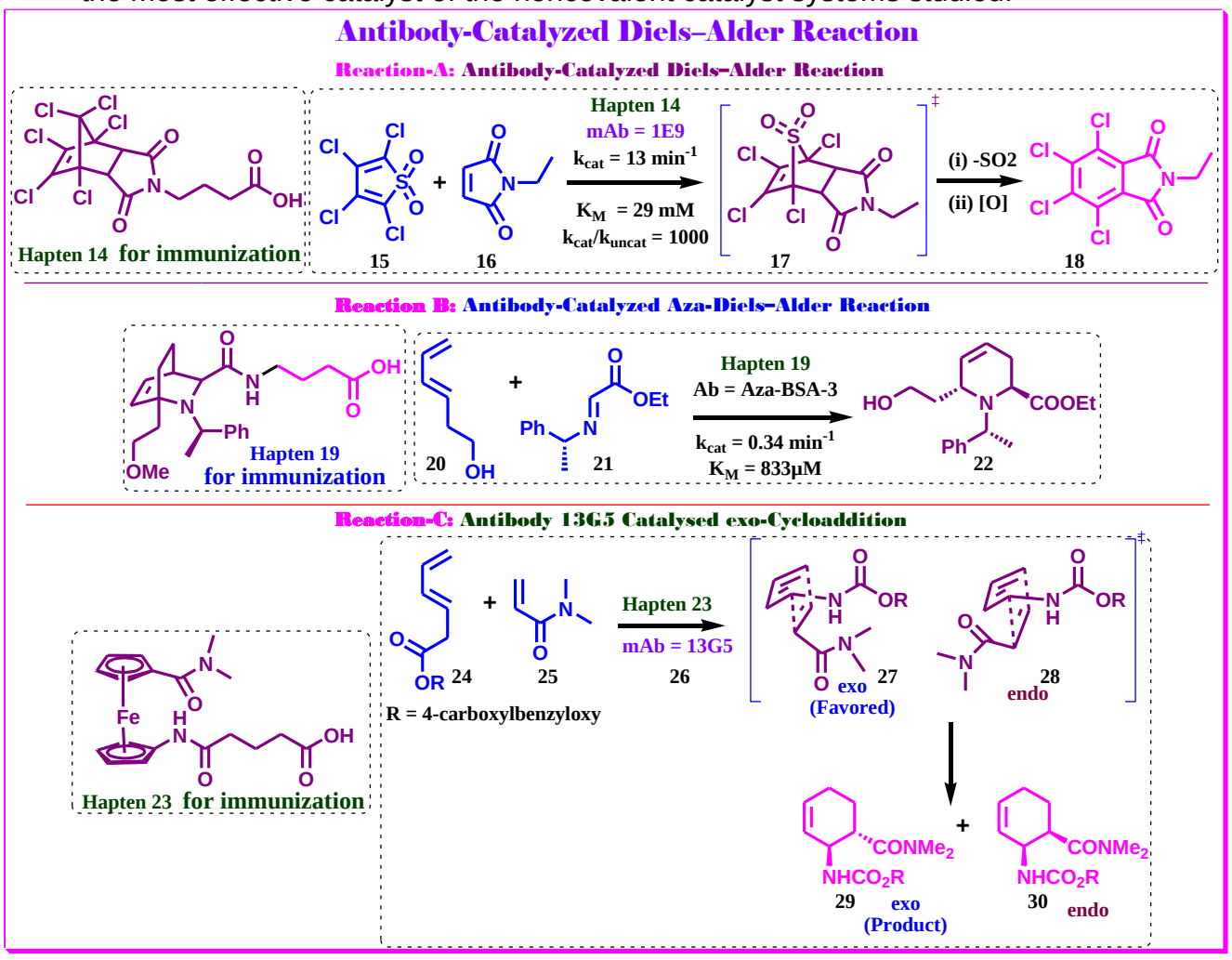


Figure 3.34: Antibody catalyzed Diels–Alder /aza- Diels–Alder reactions reactions shown and the structures of the TSA haptens.

Shi et al. have reported an antibody-catalyzed aza Diels–Alder reaction catalyzed by a polyclonal antibody Aza-BSA-3 elicited against Hapten 19, a TSA (**Figure 3.34B**).

Janda and co-workers have employed a ferrocenyl hapten 23 to produce Diels–Alderase antibodies 13G5 that catalyzed the formation of disfavoured exo-cycloaddition product upon reaction between 24 and 25 with high regio-, diastereo-, and enantio-selectivity (**Figure 3.34C**). The crystal structure study showed that the ferrocene moiety is completely buried in the antibody combining site, and the ferrocene ring rotation is restricted by the steric restraints imposed by specific hydrogen-bonding interactions with the antibody binding pocket.

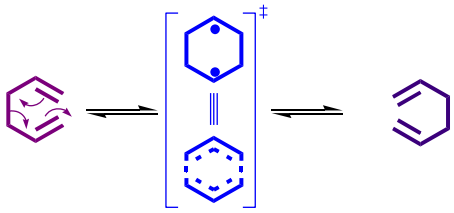
3.12.3.1.5. Antibody-catalyzed oxy-Cope rearrangement

The oxy-Cope rearrangement is a [3,3] sigmatropic rearrangement occurs *via* a highly organized chair-like pericyclic transition state. An antibody catalyst AZ-28, for such a reaction to proceed smoothly, was designed which was able to bind the substrate and orient the ground state into a productive chair-like conformation.

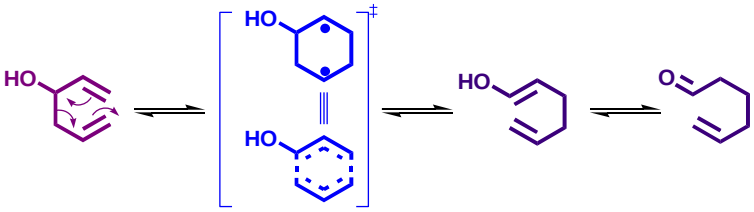
Background Information of Oxy-Cope Rearrangement

- **Geaneral form of Cope (A) and Oxy-Cope (B) reactions:**

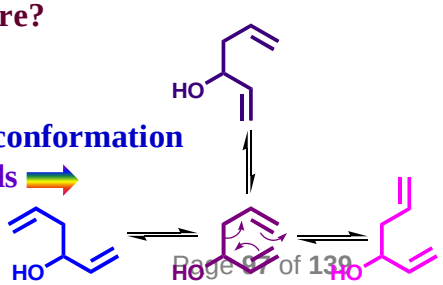
(A) Cope Rearrangement:



(B) Oxy-Cope Rearrangement:

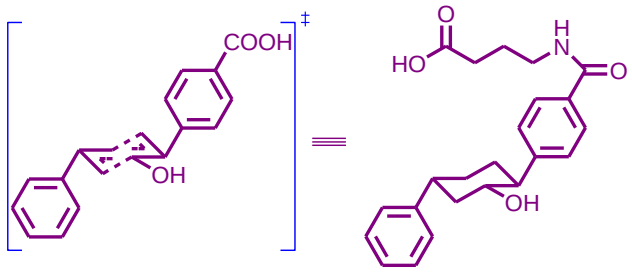


- **Oxy-Cope rearrangement is based on a diradical, cyclohexane TS intermediate**
- **It is driven by the formation of a keto-enol compound**
- **Why is this reaction slow/not possible at room temperature?**
- **It is also neither observed yet by an enzyme. Why?**
  - **Transition state is a six-membered ring in the chair conformation**
  - **In solution, there are possible rotation of sigma bonds**

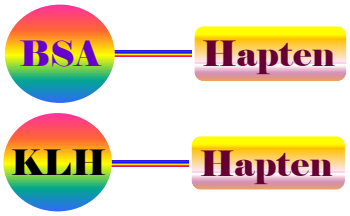


Antibody AZ-28 Catalysed Oxy-Cope Rearrangement

1. **Hapten design: Rationale and synthesis of the hapten (transition state analog):**
   - transition state is a cyclohexane intermediate
   - strong preference for aromatic rings for catalytic antibodies
   - $CONH(CH_2)_3COOH$ – is the tether to BSA/KLH



2. **Attachment of hapten to a macromolecule:**



BSA = Bovine Serum Albumin

KLH = Keyhole Limpet Hemocyanin

3. **Preparation of monoclonal antibodies:** It is already described.

Figure 3.35a: The TSA hapten and the TS of AZ-28 catalyzed oxy-Cope rearrangement.

Antibody AZ-28 was raised against the chair-like transition state analog shown in figure (**Figure 3.35**a), which catalyzes the oxy-Cope rearrangement (**Figure 3.35c**). The binding interactions of AZ-28 with the substrate revealed from crystal data are shown below in **Figure 3.35b**.

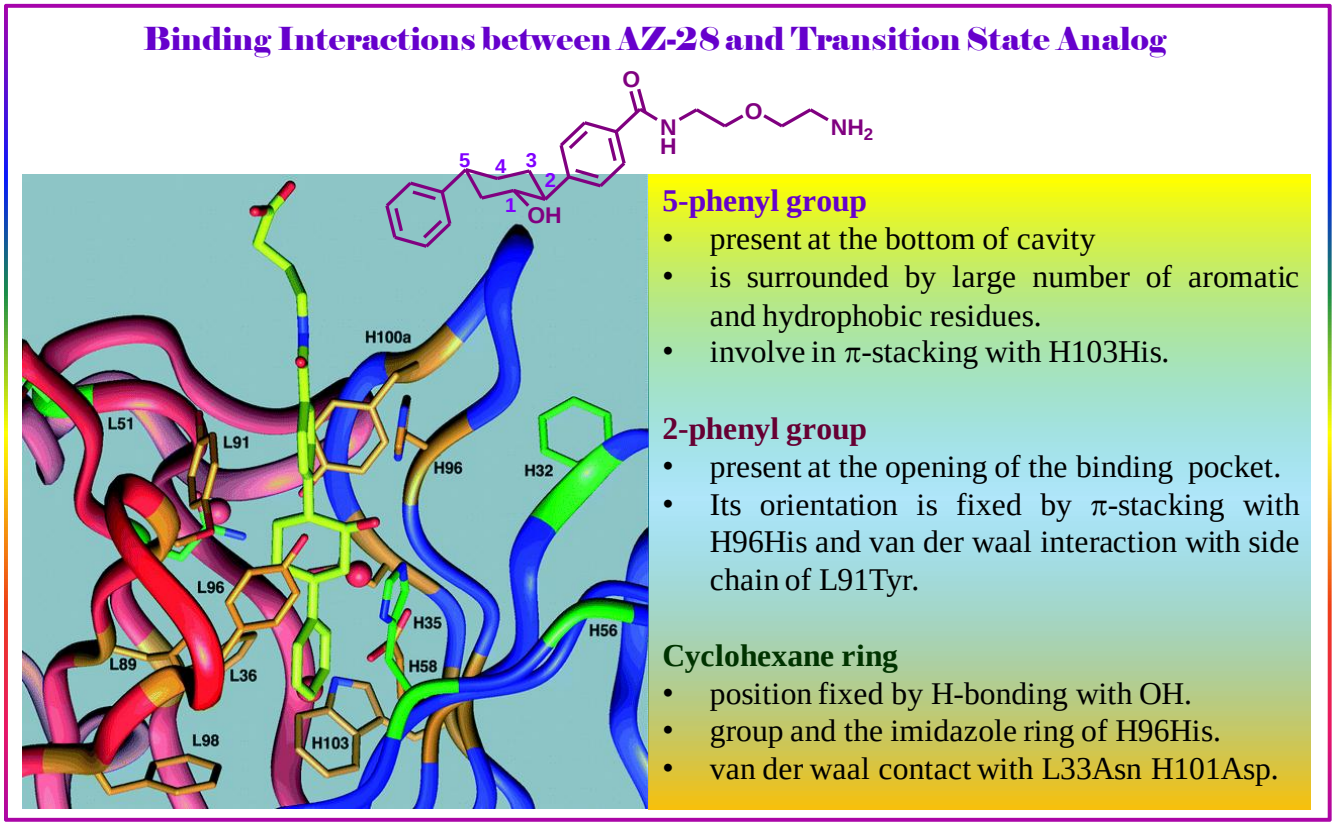


Figure 3.35b: The binding interaction between TSA hapten and the mAb AZ-28.

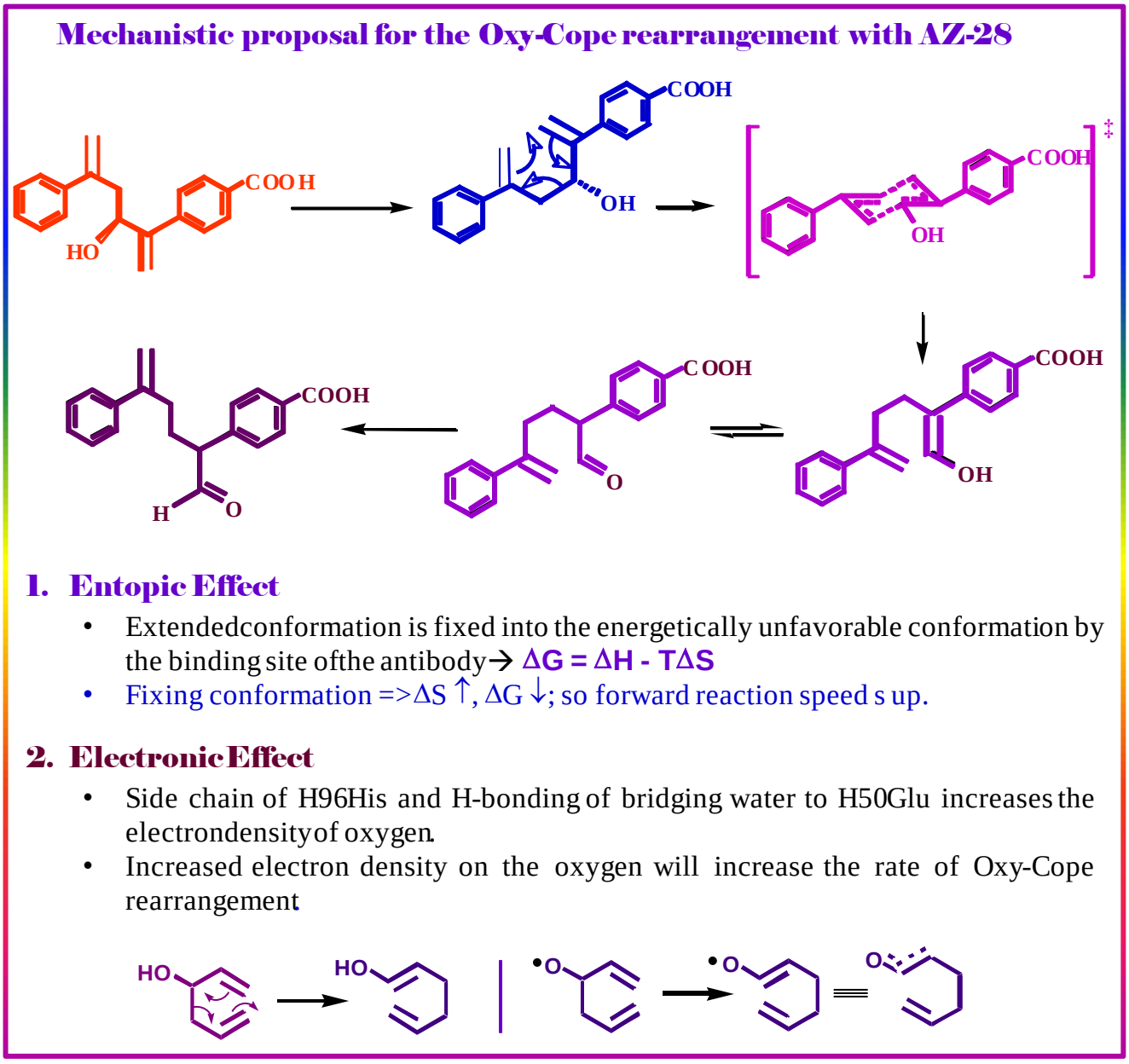


Figure 3.35c: The mechanism of oxy-Cope rearrangement catalyzed by mAb AZ-28.

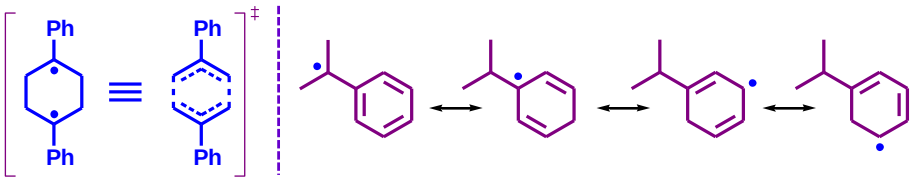
Surprisingly, the germline precursor of AZ-28, with a much lower affinity toward the eliciting hapten, accelerated the reaction 164,000-fold over the uncatalyzed background reaction. To explain the fact X-ray crystal data were collected. Thus, it was found that the TSA is fixed in a catalytically unfavorable conformation by a combination of Van der Waals and hydrogen-bonding interactions. In contrast, the active site of the germline precursor of AZ-28 appears to have a much higher degree of flexibility. The conformational flexibility in the germline antibody allows dynamic changes that lead to enhanced orbital overlap and increased rate acceleration (Figure 3.35d).

Germline Antibody is a Better than AZ-28 for Oxy-Cope Rearrangement

- AZ-28 binds the TS analog more tightly than the germline antibody, but the Germline Antibody is a better catalyst than AZ-28 for Oxy-Cope Rearrangement.

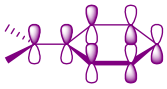
- **Reason for increased catalysis of germline antibody:**

1. Electronic Structure of T.S.:

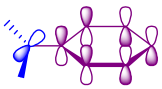


- The transition state of the Oxy-Cope rearrangement is a diradical.
- The radical can be stabilized by the aromatic group.

2. Molecular orbital reasons for increased catalytic activity:



When radical is in the same plane as aromatic ring



When radical is perpendicular to the aromatic ring

stabilization of the transition state decreases the energy requirements for catalysis

- The germline antibody fixes the TSA so that the aromatic rings are 63.2° (5-phenyl) and 57.9° (2-phenyl) to the cyclohexane framework
- AZ-28 fixes the TSA so that the aromatic rings are 81° (5-phenyl) and 85° (2-phenyl) to the cyclohexane framework.

3. Structural basis for catalytic properties of germline and AZ-28 antibody:

- Only L34 amino acid residue is at the active site-for AZ-28 → L34Asn and for Germline → L34Ser
- **Distance from L34 amino acids:** Liganded AZ-28 → 2.6 Å, Unliganded AZ-28 → 3.2 Å and Liganded Germline → 3.0 Å, Unliganded Germline → 3.7 Å
- Thus, increased flexibility of active site for germline antibody lowers the rotational barrier for the C2-phenyl.

- **Conclusion: The cause of more germline activity is due of flaw in the design of TSA. True TS possess sp^2 carbons attached to the aromatic groups. TSA have sp^3 carbons and in solution, the aromatic groups prefer to be perpendicular to the cyclohexane framework.**

Figure 3.35d: Cause of better activity of germline antibody than mAb AZ-28 for catalyzing oxy-Cope rearrangement.

3.12.3.1.6. Antibody-Catalyzed Hydride Transfer Reaction

By raising antibodies to the appropriate hapten a variety of catalytic activities may be generated. For example, the compound in **Figure 3.36**has been synthesized to mimic a proposed combined NADH–NAD cofactor product transition state of hydride transfer in dehydrogenase enzymes. Thus compounds 24 and 25 were designed and prepared as stable TSAs for the hydride transfer process between 26 and an aliphatic aldehyde 28 (**Figure 3.36**). These haptens incorporated a rigid bicyclic structure containing a 3-piperidone oxime motif. The oxime mimics the carboxamide group in nicotinamide. The piperidone is held in the boat conformation corresponding to the transition state by a three-atom lactam bridge. The aldehyde carbon in the transition state was mimicked by a methylene group in Hapten 24. In hapten 25, the same is mimicked by a sulfonyl group. Thus, theses haptens were intended to use for the generation of catalytic antibodies with the potential to act as dehydrogenase mimics for hydride transfer reaction.

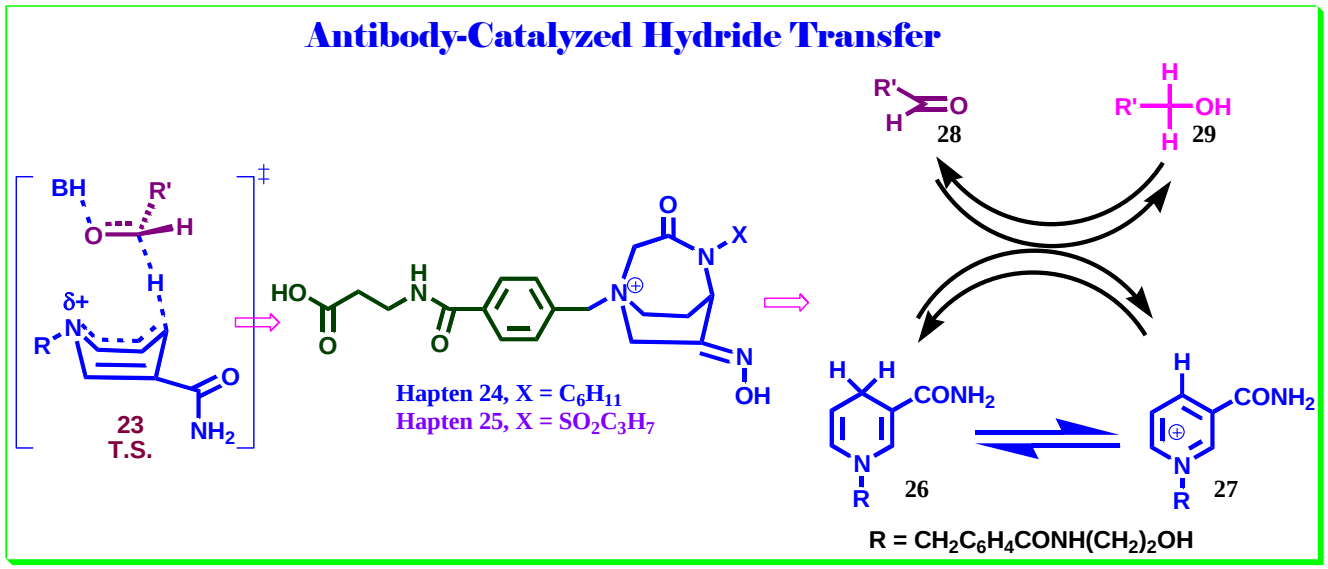


Figure 3.36: Hapten 24 and 25 were designed for the targeted hydride transfer reaction.

3.12.3.1.7. Disadvantages with TSA-Based Approaches

There are many reported instances wherein Antibody elicited against a TSA hapten is unable to catalyze the target reactions. This is due to the fact that the generation of an antibody (an immune response) upon immunization of a TSA hapten is driven by the affinity of the hapten rather than targeted catalytic activity of the produced immune response, the Antibody. Therefore, somatic mutations can raise antibodies that favor tighter hapten binding but are not suitable to perform catalysis.

Also many of the designed antibody catalysts are limited in their capability to accelerate chemical reactions as efficiently as natural enzymes in terms of efficiency [$(k_{cat}/K_M)/K_{uncat}$]. Published k_{cat}/K_M values of catalytic antibodies range from 102 to 104 $M^{-1}S^{-1}$, while those of natural enzymes range from 106 to 108 $M^{-1}S^{-1}$.

It is crucial that antibody catalysts raised against TSAs have to release the product to be considered efficient, Furthermore, in a number of cases it was found that because of high affinity of the antibody to bind the formed product tightly (which is again due of structural similarity of the product to the TSA haptens used in immunization), the products of the reaction can not be released. Therefore, the efficiency of the antibody is very low and can not be useful, which is a major case for many hydrolytic antibodies.

Another possibility for the poor performance of some transition state analog-based antibody catalysts may be an inability to design a stable organic compound that could reproduce the geometric criteria such as fractional bond orders, extended bond lengths, expanded valences, distorted bond angles, and charge distributions in the short-lived structure of a transition state.

The high catalytic proficiency of natural enzymes is due to the fact that the substrates remain bound buried in their catalytic machinery. However, in antibody catalysis, the moieties of the bound haptens that play a major role in catalytic activity and mimic the transition state are often positioned near the entrance of the antibody-combining site. This disparity in the overall architecture of natural enzymes and catalytic antibodies is undoubtedly a factor in the lower catalytic proficiency of the catalytic antibodies.

Therefore, antibody structure itself places limitations on the kind of reactions agreeable to catalysis. Thus, many other strategies have been developed to generate catalytic antibodies which act like enzyme as in general base and covalent catalysis, proximity effects, and the use of strain. We are going to exemplify few of such examples.

3.12.3.2. ‘Bait-and-switch’ Strategy

Based on the fact that a charged small molecule can mimic a charged amino acid residues in the antibody-binding site, a new approach for hapten design has been developed to expand the scope of antibody catalysis. In this strategy a point charge is placed on the hapten in close proximity to a chemical functional group or a charged substituent is placed that is expected to transform the corresponding substrate to product. The haptenic charge is then expected to induce a complementary charge at the active site of the antibody. The charged amino acid residues thereby are chosen to contribute to catalysis as general-acid/base or nucleophilic catalysts. Since the haptens designed according to this strategy serve as ‘bait’ for eliciting catalytic functions during the immunization process, which is then ‘switched’ for the substrate, the strategy is called **‘bait-and-switch’**.

Using this strategy, Antibody MATT.F-1, raised against a quaternary ammonium hapten 31 whose TSA hapten is 30, the hydrolysis of phosphodiester bonds (which are often found in DNA and RNA and is a reaction of significant importance in living systems) was achieved with high proficiency (**Figure 3.37**). The Designed Hapten 31 incorporate a general base in an antibody binding site proximal to the 2′ hydroxy of substrate 32 to facilitate nucleophilic attack of this hydroxyl group on the adjacent phosphoryl center. Thus, here the transition state mimicry is sacrificed and replaced by a point charge. MATT.F-1 has a catalytic proficiency [$(k_{cat}/K_m)/k_{uncat}$] of 1.6 × 107 M^{-1}, which is higher than that reported for antibody 2G12, elicited to the transition state analog hapten 30. However, the proficiency of MATT.F-1 is only three times lower than that of the naturally occurring enzyme RNAseA for the same substrate for same hydrolysis.

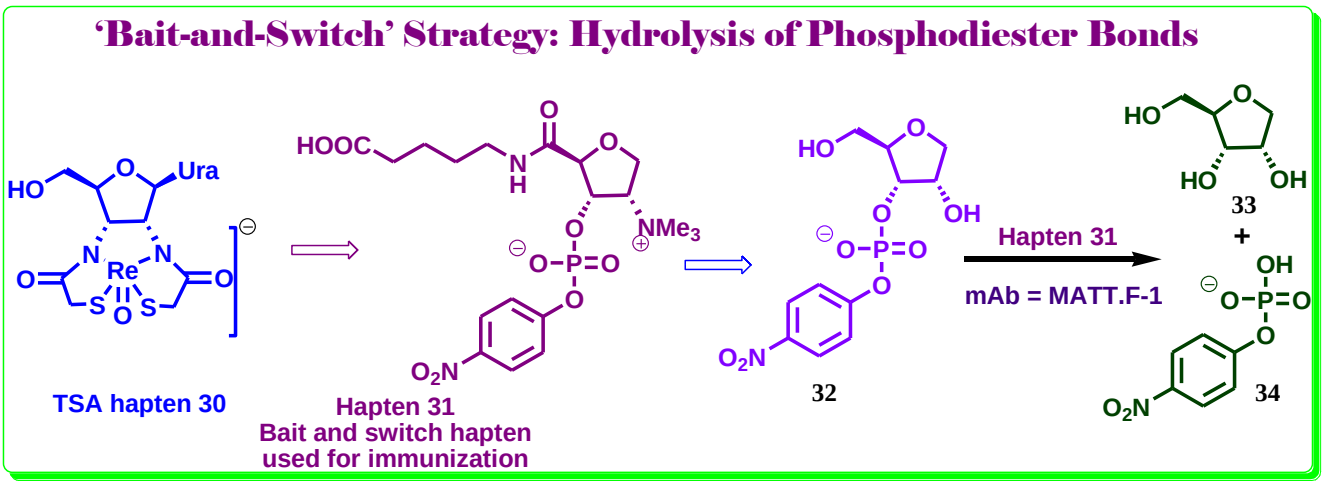


Figure 3.37: Hapten 31 (Bait and Switch) and the hydrolysis of phosphodiester bond catalyzed by mAb MATT.F-1.

3.12.3.3. The Reactive Immunization Strategy

It is clear now that both the TSA and bait-and-switch strategies to generate catalytic antibodies rely for the most part on chemically inert antigens. However, Antigens that mimic the geometric and/or electronic features of a reaction's transition state can enable the generation of catalytic antibodies. Therefore, a new hapten design strategy—reactive immunization—may provide a chance for eliciting catalytic antibodies to realize the catalytic efficiency of natural enzymes through the use of reactive immunogens.

Thus, to explore this strategy, in 1995 Janda, Lerner and co-workers designed an organophosphorus diester hapten 35, as the primary reactive immunogen to elicit catalytic antibody to perform a chemical reaction in the antibody-combining site during immunization. This hapten can be either hydrolyzed at physiological pH or trapped by a nucleophile at the B-cell level of the immune response affording the monoester 36, an analog of the transition state (**Figure 3.38A**). Nineteen monoclonal antibodies (mAbs) were isolated, out of eleven were able to catalyze the target acyl-transfer reaction, hydrolysis of phosphonate diester 37 to

generate the monoester 38 (**Figure 3.38C**). Amongst these eleven catalytic antibodies, SPO49H4 demonstrated the best catalytic activity. It effectively catalyze the hydrolysis of the activated ester 39 to yield carboxylic acid 40 with a k_{cat} of 31 min^{-1} and a rate acceleration (k_{cat}/k_{uncat}) of 6700 at pH 8.0.

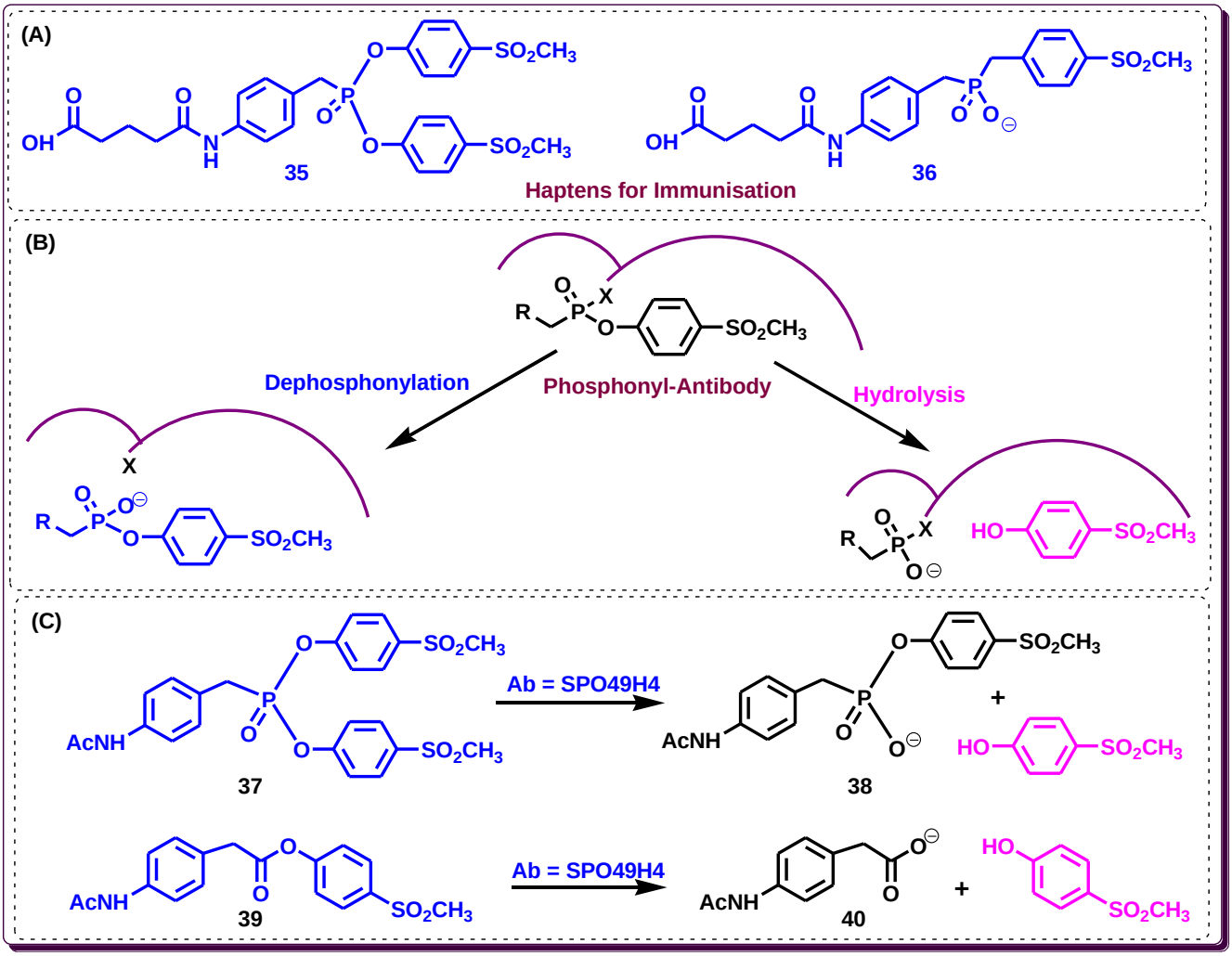


Figure 3.38: (A-B) Haptens and possible chemical reaction that can take place in the antigen combining site when the antibody reacts with the phosphonate diester [X = nucleophile in the antibody]. (C) Reactions catalyzed by catalytic Ab SPO49H4.

The first direct comparison between reactive immunization and transition state analog hapten for catalytic antibody production was demonstrated by comparing esterase antibodies elicited against a TSA, phosphonate monoester 45, with the hapten raised by a reactive immunogen, phosphonate diester 44. Thus, hapten 44 was initially design for the purpose of resolving a racemic mixture of naproxen esters. Antibody 15G2 generated by using hapten 44 (reactive immunization) catalyzed the homochiral production of the anti-inflammatory agent Naproxen,

rac-42, from 41. The S-(+)-enantiomer 42a of naproxen is formed 28 more times than the R-(−)-enantiomer 42b (**Figure 3.39**). This antibody catalyzed the hydrolysis of S-(+)-41a and gave S-(+)-42a with a k_{cat} = 28 min^{-1}, K_m = 300 μM at pH 8.0, and k_{cat}/K_m = 9.3 × 10^4 M^{-1} s^{-1}. On the other hand, antibody 6G6, raised against the transition state analog hapten 45, catalyzed the same reaction with a k_{cat} = 81 min^{-1}, K_m = 890 μM at pH 8.0, and k_{cat}/K_m = 4.5 × 10^4 M^{-1} s^{-1}. The transition state analog approach provided good biocatalysts in terms of turnover numbers and enantiomeric discrimination, but it suffers from varying degrees of product inhibition by phenol 43. Therefore, the antibody generated via Reactive immunization are more efficient and do not suffer from product inhibition.

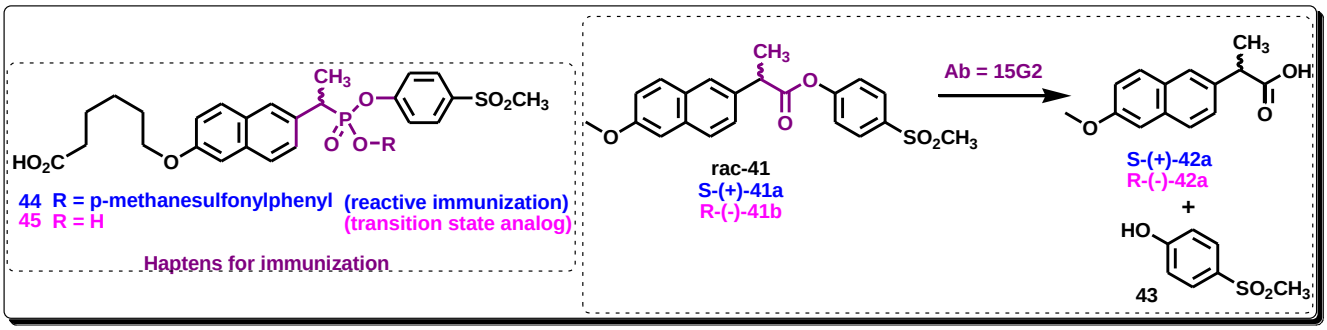


Figure 3.39: Kinetic resolution by catalytic antibodies, Ab 15G2.

The reactive immunization strategy has also been utilized in the aldol reaction. Two aldolase antibodies, 38C2 and 33F12, were elicited by hapten 46, equipped with a reactive β-1,3-diketone functionality. The β-1,3-diketone moiety demonstrated its distinct ability to trap a lysine side chain amine affording an enaminone 48 through Schiff base 47, which directly participates in the mechanism of the aldol reaction in the active site of the antibody (**Figure 3.40**). The two antibodies, 38C2 and 33F12, obtained by reactive immunization performed extraordinary catalysis of the aldol reaction between acetone and the aldehyde 49, with high catalytic proficiency (**Figure 3.40**).

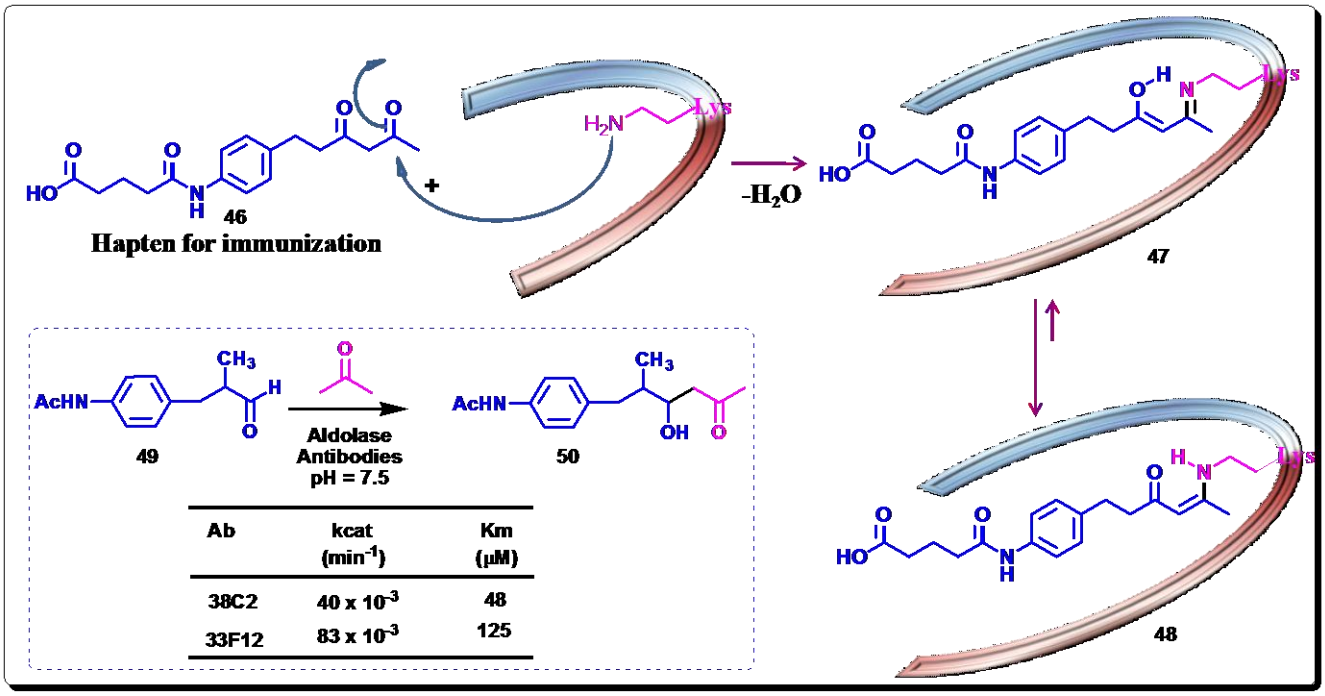


Figure 3.40: Mechanism of trapping the essential ε-amino group of a lysine residue (LysH93) in the antibody's binding pocket by using the 1,3-diketone hapten 46 and Aldol reaction catalyzed by mAb 38C2 and 33F12.

Zong *et al.* employed reactive immunization strategy in combination with transition state theory to find more efficient aldolase antibodies. Thus, on the basis of hapten 46, a hybrid, hapten 51, was designed, employing a sulfone functionality to establish the tetrahedral motif that present in the transition state, and a β-diketone for trapping a lysine side chain at the active site (**Figure 3.41**). Two aldolase antibodies, 93F3 and 84G3 were isolated. In the aldol reaction of 54 with 3-pentanone, antibody 93F3 provided syn-aldol 55 with 90% de and 99% ee, while antibody 38C2, elicited to hapten 46 afforded only 62% de and 59% ee. Antibodies 93F3 and 84G3 showed a 103-fold increase in proficiency over the antibody 38C2 for the kinetic resolution of (±)-56(**Figure 3.42**) [Zhong, G. F.; Lerner, R. A.; Barbas, C. F. *Angew. Chem., Int. Ed.* **1999**, *38*, 3738].

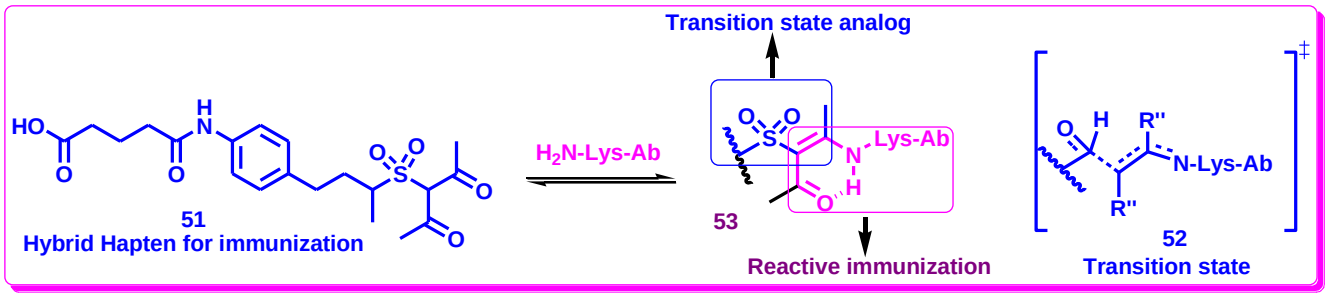
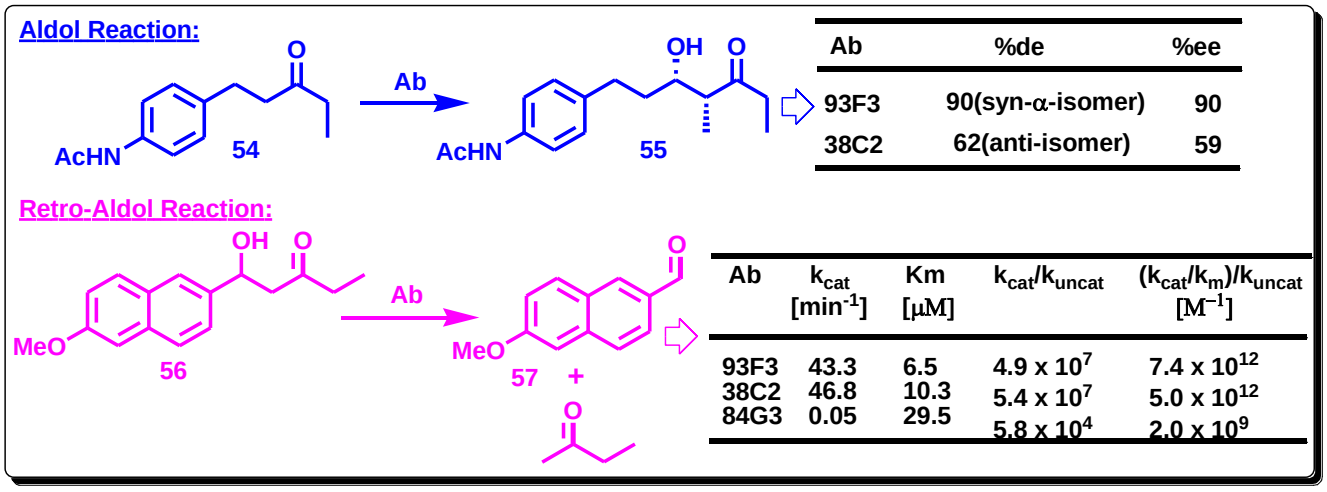


Figure 3.41: The hybrid hapten 51 designed from the presume transition state.



Ab	%de	%ee
93F3	90(syn-α-isomer)	90
38C2	62(anti-isomer)	59

Ab	k_{cat} [min^{-1}]	Km [μM]	k_{cat}/k_{uncat}	$(k_{cat}/k_m)/k_{uncat}$ [M^{-1}]
93F3	43.3	6.5	4.9×10^7	7.4×10^{12}
38C2	46.8	10.3	5.4×10^7	5.0×10^{12}
84G3	0.05	29.5	5.8×10^4	2.0×10^9

Figure 3.42: Comparison between 93F3, 84G3, and 38C2 for aldol and retro-aldol reactions.

Mechanism-based inhibitors covalently react with the active site in target proteins and inhibit their activities. Therefore, such inhibitors provide a wealth of information to guide the design of haptens for immunization for generating catalytic antibodies. Penam sulfones are potent mechanism-based inhibitors of β-lactamase. They by forming an acyl-enzyme intermediate inactivate the enzyme. Therefore, this efficiency of sulphones inspired the design of the sulfone hapten 58 targeting the hydrolysis of the lactam functionality built in the substrate 59. Immuno conjugate 58-KLH was immunized, and a scFv library was constructed using the spleen cells of immunized mice. Screening of the library yielded two scFv antibodies, FT6 and FT12. These antibodies catalyzed hydrolysis of 59 with rate accelerations (k_{cat}/k_{uncat}) of 5200 and 320, respectively (**Figure 3.43**).

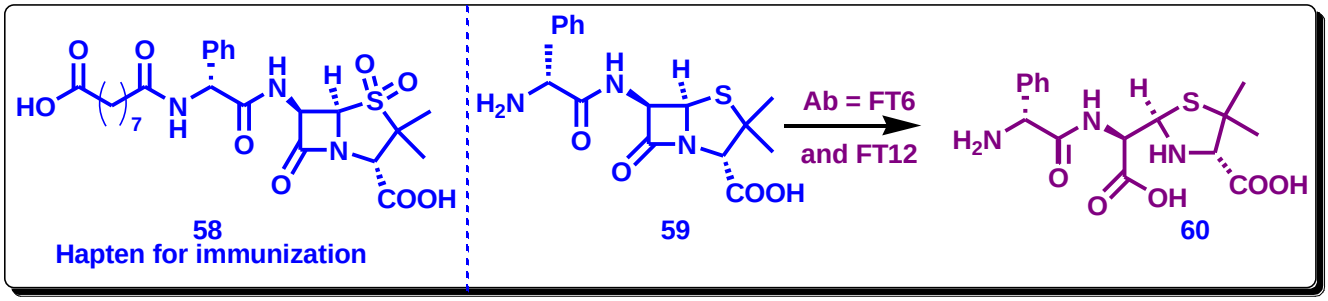


Figure 3.43: Reactive hapten 58 and the reaction catalyzed by mAb FT6 and FT12.

3.12.3.4. Cofactor Approaches

Metal-coordinated enzymes are everywhere in nature. A metallic species at the active site of an enzyme often plays a critical role in the reaction pathway by enhancing substrate selectivity and accelerating reaction rates and are the cofactor of the enzyme. Inspired by the efficiency of metalloenzymes, several efforts have been put forth toward the development of improved antibody catalysts with metals at the active site. Thus, antibody aldolase 38C2 was

chosen as the parent antibody by several groups for the development of novel catalytic antibodies by the employment of cofactors. Nicholas et al. have employed bis-imidazolyl ligand coordinated copper complexes a cofactor which coordinate with LysH93 residue of 61 equipped with a reactive succinic anhydride moiety and $CuCl_2$. This semisynthetic metalloantibody, **38C2-62-$CuCl_2$**, catalyzed the hydrolysis of picolic acid ester 62 in aqueous buffer under physiological conditions. This study exemplified that modification of the active site by a metal-coordinated ligand could alter the catalytic nature of the parent antibody, affording a catalyst with very different catalytic activity (**Figure 3.44**).

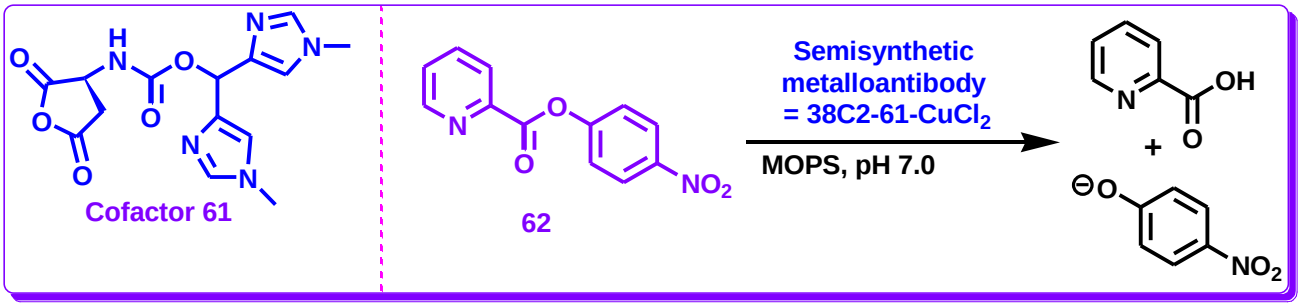


Figure 3.44: Cofactor 61 and the ester hydrolysis catalyzed by semisynthetic metalloantibody 38C2-61-$CuCl_2$.

Pyridoxal 5′-phosphate (PLP, 63) has been shown to be an effective cofactor for antibody-catalyzed aldol and retro-aldol reactions. Aldolase antibody 10H2, elicited to hapten 64, and combined with cofactor PLP 63, catalyzed the aldol reaction between glycine and aldehyde 65, with a rate acceleration of double the background reaction where no PLP was applied (**Figure 3.45a**). This incorporation of PLP also improved the rates of the retro-aldol reactions of the threo- and erythro- isomers with rate enhancements of 4-fold and 2.5-fold, respectively (**Figure 3.45b**).

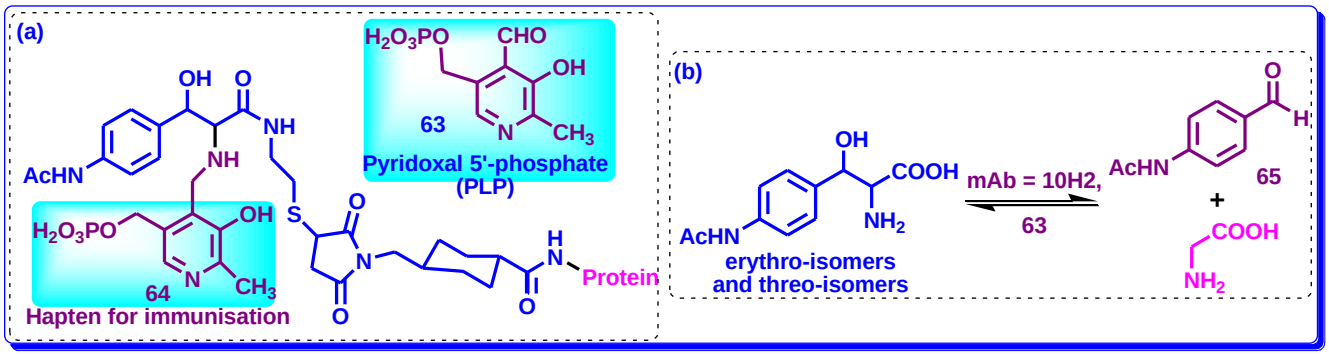


Figure 3.45: (a) Structure of PLP and hapten conjugated with carrier protein, (b) aldol and retro-aldol reactions catalyzed by mAb 10H2 with PLP 63.

Metalloporphyrins are well known in the research field of biological oxidation processes. Its application as a cofactor for a catalytic antibody was recently investigated. Thus, antibody SN37.4, elicited against a water soluble tin(IV)

porphyrin 66 showed its oxidative activity upon assembly with a ruthenium(II) cofactor 67. This antibody-metalloporphyrin assembly showed enzymatic characteristic and transfer oxygen to substrate 68 with high enantioselective (**Figure 3.46**).

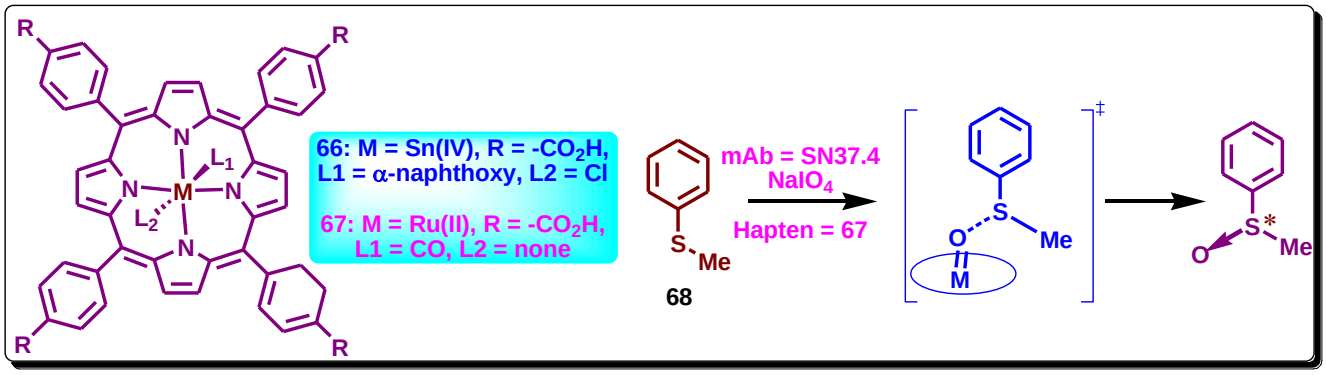


Figure 3.46: Metalloporphyrine haptens and the shown oxidation reaction catalyzed by antibody SN37.4.

3.12.3.5. Conclusion

From the above discussions and examples, it is clear that catalytic antibodies are efficient similar to the proficiency of natural enzymes in successful chemical transformation. Therefore a great attention has been drawn to this field of research. A large number of antibodies that are able to catalyze a variety of chemical processes have been investigated. Many advances based on the concept of catalytic antibodies as first described by Jencks, has been achieved. The ability to catalyse a chemical transformation stereospecifically and regiospecifically would offer the greatest immediate potential for their exploitation in the pharmaceutical and fine chemical industries for the synthesis and manufacture of new and purer drug candidates.

Antibodies that catalyze aminoacylation reaction could be used for facilitating aminoacylation of tRNAs with unnatural amino acids, an exciting focus towards expansion of genetic code and studies of protein biosynthesis. Efforts toward the exploitation of catalytic antibodies as therapeutics is the current research focus. It is also a hope that in near future potent catalytic antibody can be generated to treat diseases like HIV infection and Alzheimer's disease, cancer. Phage-based screenings, selections and rational design can help one to engineer antibodies with anticancer activity and metallo-antibodies with hydrolytic activity.

Thus, these advancements, as well as future effort in this field of research will facilitate the discovery of more efficient catalytic antibodies that might provide synthetic chemists with novel tools to target challenging synthetic problems.

Clearly, the demonstration of the generality of catalytic antibodies and their role in understanding fundamental aspects of catalysis has generated excitement and opened new horizons of scientific and industrial opportunity that bridge enzymology, immunology and chemistry.

3.13.Enzyme Models: Biomimetic Polyene Cyclization

3.13.1. The Enzyme Models

3.13.1.1. Introduction: Definition and Importance of Enzyme Models

The synthetic organic small molecules that contain one or more features of enzymatic systems and mimic the enzymatic function on a much simpler level and simpler way are called Enzyme Models. They are smaller and structurally simpler than enzymes.

To analyze a particular factor responsible for the catalytic efficiency of the enzyme within the large complex biological system is a cumbersome job that requires knowledge of each of the components that would contribute to the overall catalytic activity of the enzymes. Instead, with appropriate synthetic models, it is possible to estimate the relative importance of each catalytic parameter in the absence of those not under consideration.

One major advantage of the use of “artificial structures” for modeling enzymatic reaction is that the compounds can be manipulated precisely for the study of a specific property i.e. to mimic a specific function of an enzyme whose model it will be. The structure of the model may be further refined by combining those features that contribute to reach the efficiency of the enzyme.

Thus, with the tools of synthetic chemistry, it becomes possible to construct a “miniature enzyme’’ which lacks a macromolecular peptide backbone but contains reactive chemical groups/reactive core correctly oriented in the geometry dictated by an enzyme active site. This approach is often also referred to as the biomimetic chemical approach to biological system (see Module 1 for details). Therefore, biomimetic chemistry represents the field that attempts to imitate the acceleration and selectivities characteristic of enzyme-catalyzed reactions. It is hoped that such an approach will eventually bridge or at least reduce the gap between the known complex structures of organic biomolecules and their exact function in life. In order to do this, many factor related to the mechanism of action of a particular enzyme must be known such as (a) the structure of the active site and the enzyme–substrate complex, (b) the specificity of the enzyme and ability to bind to the substrate, and (c) the kinetics for the various steps and a knowledge of possible intermediates.

Enzymes are complicated molecules and only a few mechanisms have been definitely established. This is one of the reasons why model systems are necessary. Among the functional groups found on polypeptide chains, those generally involved in catalytic processes are the imidazole ring, aliphatic and aromatic hydroxyl groups, carboxyl groups, sulphydryl groups, and amino groups. It is wondering how such a limited number of functional groups can participate in the large variety of known enzymatic reactions. How can the rate of the enzymatic reactions be accounted for in a mechanistic way? These are the fundamental questions we must have to take care during the planning of designing of a bioorganic model of an enzyme.

3.13.1.2. Criteria to Design a Good Enzyme Models

A model also can represent general features of more than one enzyme too. In general, the requirements necessary for the design of a good enzyme models are:

(a) The model should provide a good (hydrophobic) binding site for the substrate to play the noncovalent interactions which are the key interactions to bring biological flexibility and specificity for a substance and for a reaction.
(b) Electrostatic and hydrogen bonds play a major role in binding and thereby in forwarding the reaction. Therefore, the model should provide the possibility of forming electrostatic and hydrogen bonds to help substrate bind in the properly attached to the model to effect the reaction.
(c) To mimic the active site of the enzyme, carefully selected catalytic groups have to be properly attached to the model to effect the reaction.
(d) The structure of the model should be rigid and well defined, particularly with respect to substrate orientation and stereochemistry. All this from a-d will allow good complementary intermolecular interactions as well as a chiral discrimination.
(e) To mimic the full enzyme which works at a particular physiological pH, and temperature, the model should preferably be water soluble and catalytically active under physiological condition of pH and temperature.
(f) To be a good model, it should provide a reasonable simulation of the enzyme mechanism,
(g) It is also necessary that the model should lead to an explanation of the observed rate enhancement in terms of structure and mechanism.
(h) At last, all the information obtained with enzyme models must ultimately be compared and test to the in vivo enzymatic system under study in order to correlate the bioorganic models to the real natural system.
(i) The Model, enzyme like catalyst, must also obey Michaelis-Menten kinetics (saturation behaviour), lead to a rate enhancement, and show bi- and/or multifunctional catalysis.

Taking into consideration of these criteria one can construct a model matrix that can bring catalytic group and substrates together to a fruitful mimicking the enzyme function. The so designed matrix with the active core, can involve in the binding process to raise the ground state energy of the substrate by rigidification and bond distortion like an similar way as an enzyme do. Also, proper stereochemistry between the model catalyst and the substrate will result in better specificity and efficiency of reaction. Biomodels of many reactions are presented in a tabular form in Module 1.

3.13.1.3. Examples of Enzyme Models

Many enzyme models were studied which includes:

(a) Hydrolytic model
(b) Models with proteolytic activities
(c) Models with glycolytic activities
(d) Nucleolytic enzyme models
(e) Assembly of macromolecules such as proteins and nucleic acids
(f) Structures resembling drug receptors may be incorporated into synthetic membranes, facilitating studies of these receptors without immunological and toxic complications.
(g) Furthermore, the ability of membranes to segregate charged species finds commercial use in systems for energy storage or hydrogen generation.

3.13.2. Biomimetic Polyene Cyclization

3.13.2.1. Introduction to Biomimetic Synthesis

Biomimetic Synthesis or Biogenetic Type Synthesis: Biomimetic synthesis may be defined as the design and execution of laboratory reaction based upon established or presumed biochemical transformation. This implies the development of chemical transformation new to the nonbiological area and the elaboration of elegant total synthesis of various natural product precursors.

- **Van Tamelen's Definition:** A specific reaction or a sequence of reactions that mimic a proposed biological pathway is defined as biomimetic synthesis.
- **Miguel A. Sierra's Definition:** The term biomimetic synthesis is also used to describe a sequence of reaction carried to support a biogenetic hypothesis.

Biosynthesis or Biogenetic Synthesis: The reaction or reaction sequence occurred in organism or its immediate environment will be viewed as biosynthesis---Dirk Trauner.

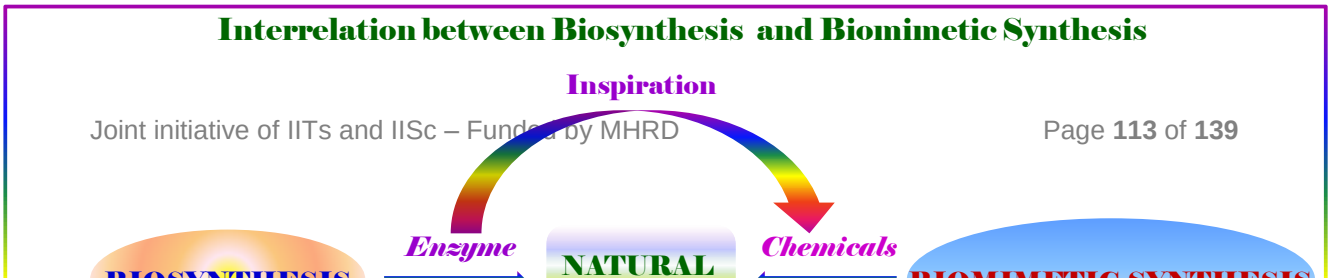
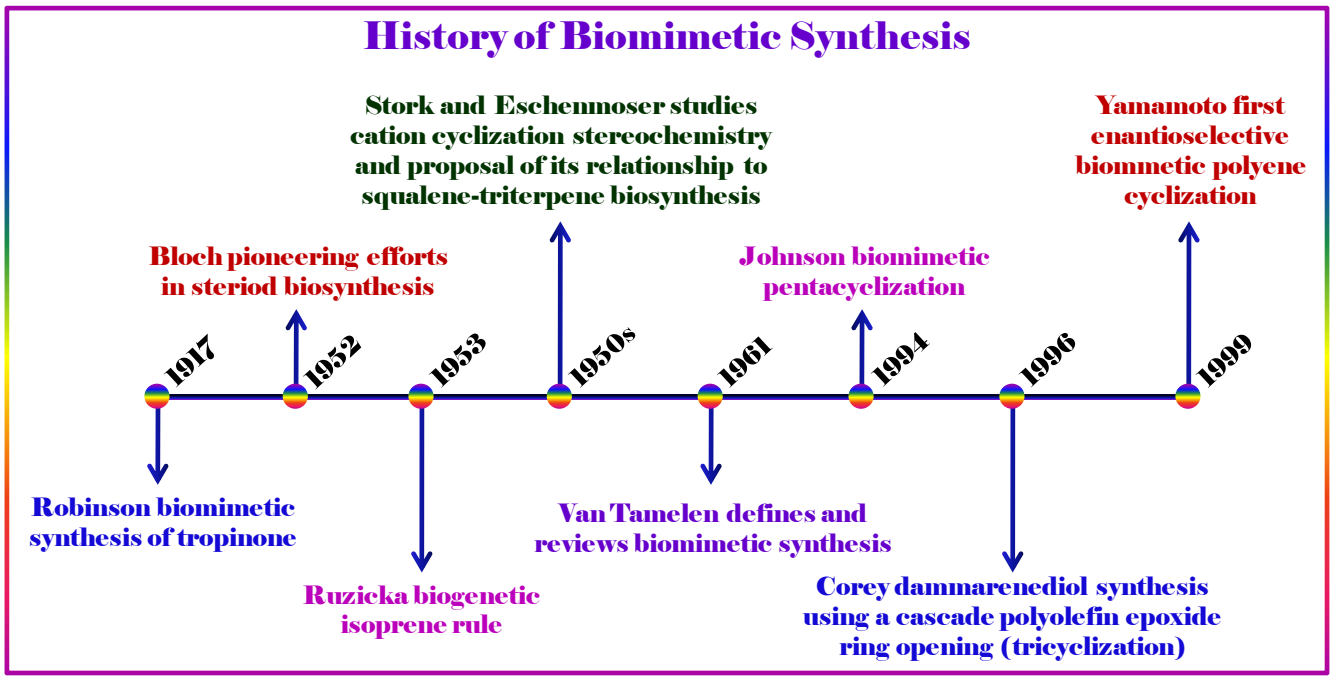
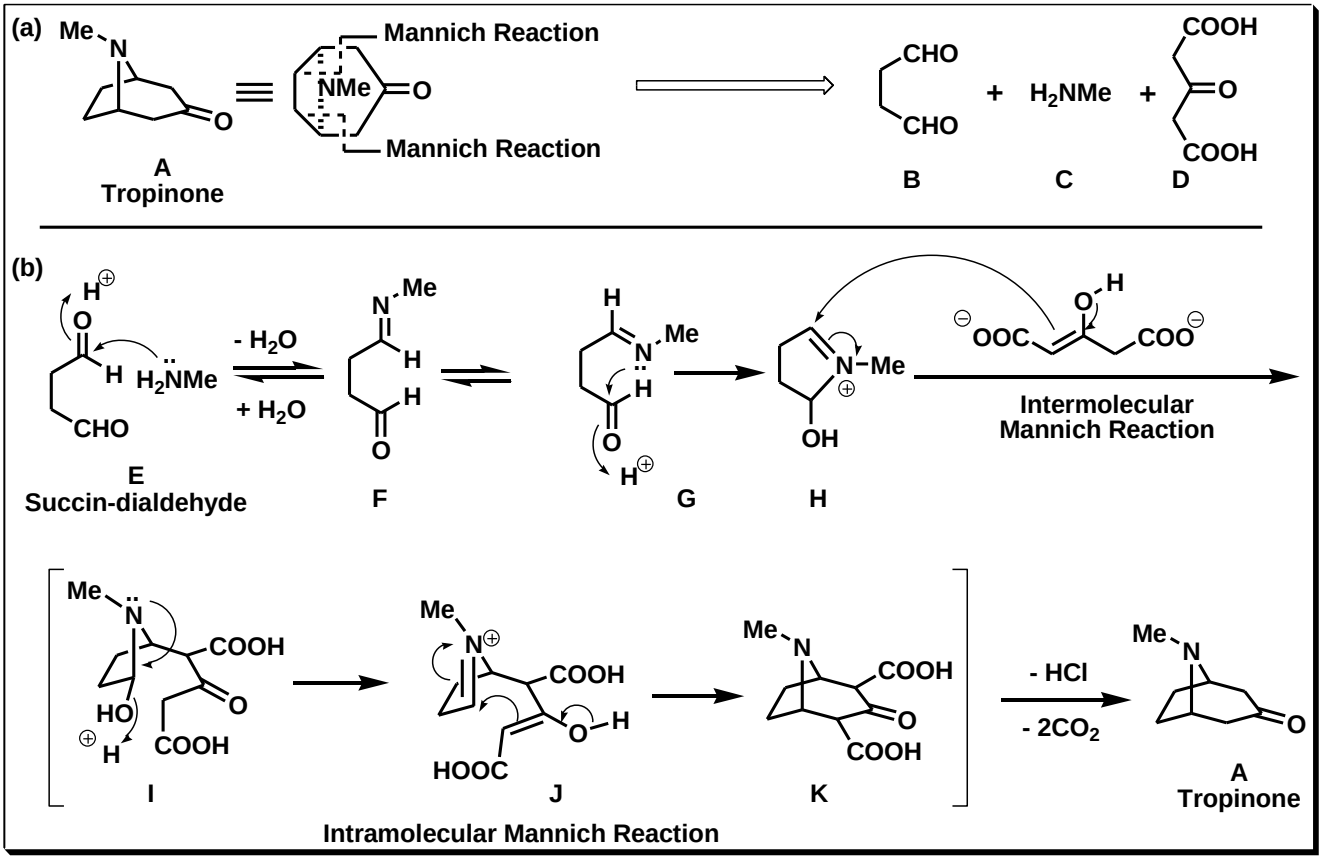


Figure 3.47: The interrelation between Biosynthesis and Biomimetic Synthesis.

3.13.2.2. The Landmarks in the History of Biomimetic Synthesis



3.13.2.3. The Beginning of Biomimetic Synthesis--*Robinson's Biomimetic Synthesis of Tropinone* [Retrosynthetic analysis of tropinone and total synthesis (Robinson, 1917)]



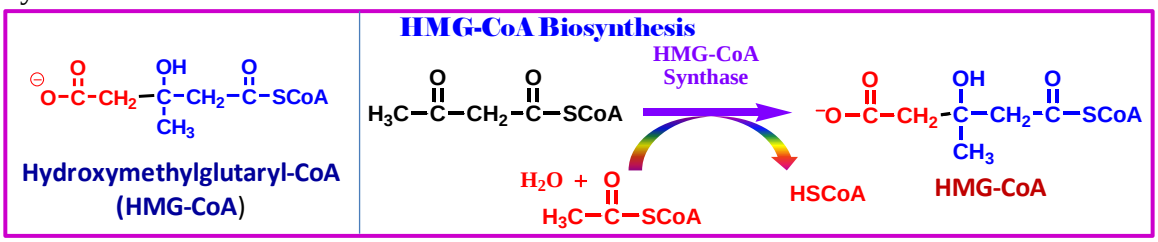
3.13.2.4. Biosynthesis of Squalene

Squalene is the precursor of sterols and polycyclic triterpenes. In the 1950s, G. Stork and A. Eschenmoser proposed that the biogenetic conversion of squalene to lanosterol involved a synchronous oxidative cyclization pathway. The transformation is acid catalyzed and proceeds through a series of carbonium ions to allow the closure of all four rings. There is now ample evidence that the first step is the selective epoxidation of the –double bond to from 2,3-oxidosqualene. The following steps involved in the biosynthesis of squalene and then to lanosterol.

HMG-CoA– Precursor for Squalene/Cholesterol Biosynthesis

HMG-CoA Biosynthesis

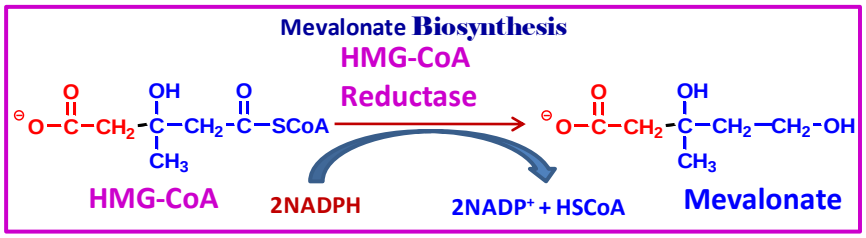
- **Hydroxymethylglutaryl-coenzyme A (HMG-CoA)** is the precursor for cholesterol synthesis.



- **HMG-CoA** is also an intermediate on the pathway for synthesis of **ketone bodies** from acetyl-CoA.
- **HMG-CoA** is formed by condensation of acetyl-CoA & acetoacetyl-CoA, catalyzed by **HMG-CoA Synthase**.

Mevalonate Biosynthesis

- **HMG-CoA Reductase** catalyzes production of **Mevalonate** from HMG-CoA.
- This enzyme is highly **regulated** and the target of pharmaceutical intervention.

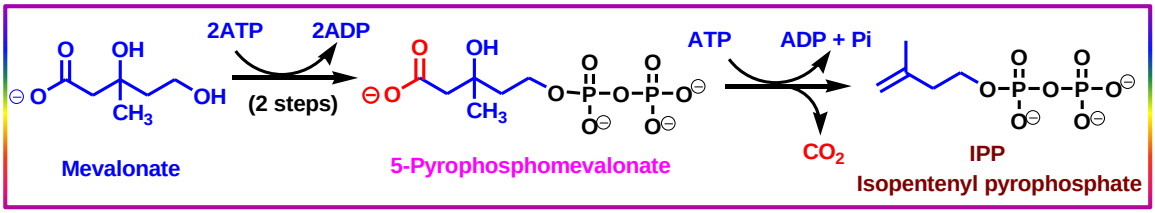


- The **carboxyl** of HMG that is in ester linkage to the CoA thiol is reduced to an **aldehyde**, and then to an **alcohol**.
- **NADPH** serves as reductant in the 2-step reaction.
- **Mevaldehyde** is thought to be an active site intermediate, following the first reduction and release of CoA.
- Mevalonate formation from HMG-CoA, is the **rate-limiting step** for cholesterol synthesis.

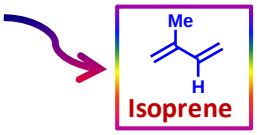
Biosynthesis of IPP & DMAPP

Synthesis of Isopentenyl Pyrophosphate:

- **Mevalonate** is phosphorylated by 2 sequential P_i transfers from ATP, yielding the **pyrophosphate** derivative.

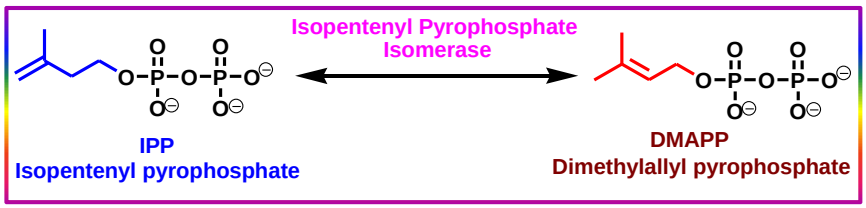


- ATP-dependent decarboxylation, with dehydration, yields **isopentenyl pyrophosphate**.
- **Isopentenyl pyrophosphate** is the first of several compounds in the pathway that are called as **isoprenoids**, by reference to the compound **isoprene**.



Isomerisation:

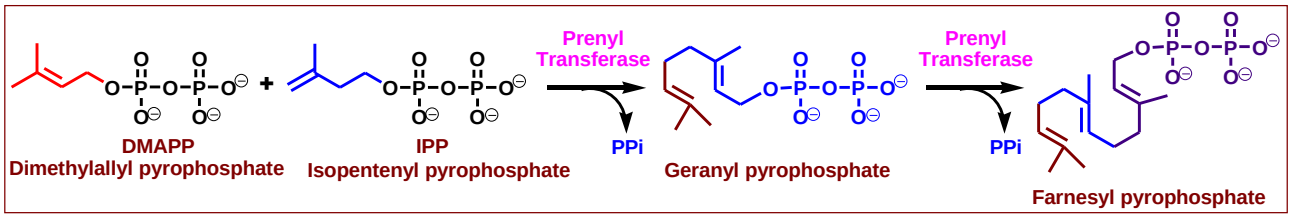
- **Isopentenyl Pyrophosphate Isomerase** inter-converts isopentenyl pyrophosphate & **dimethylallyl pyrophosphate** → Mechanism: protonation followed by deprotonation.



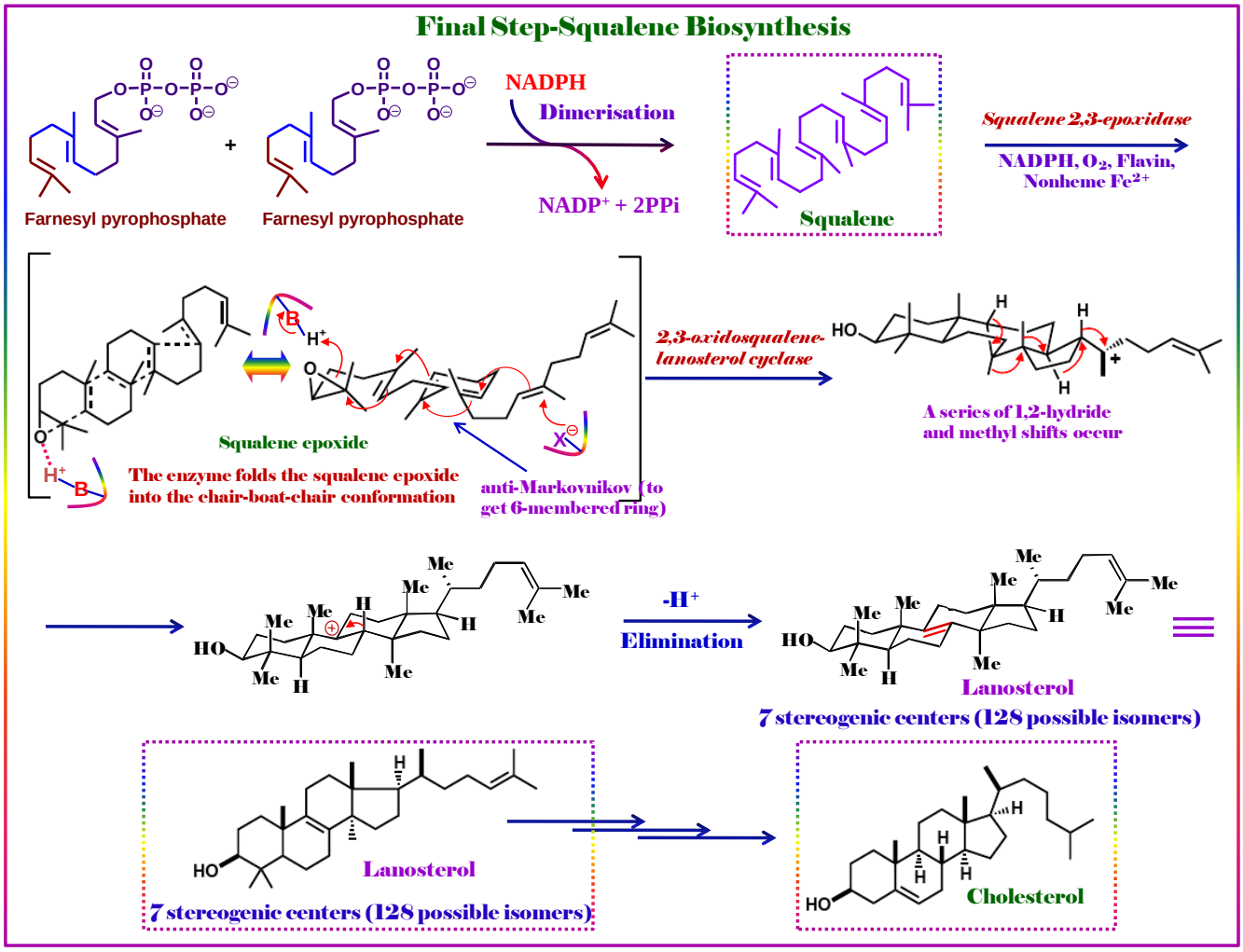
Biosynthesis of IPP & DMAPP

Prenyl Transferase catalyzes head-to-tail condensations:

- Dimethylallyl pyrophosphate & isopentenyl pyrophosphate react to form **Geranyl pyrophosphate**.



- Condensation with another isopentenyl pyrophosphate yields **Farnesyl pyrophosphate**.
- Each condensation reaction is thought to involve a reactive **Carbocation** formed as **PP_i** is eliminated.



3.13.2.5. Case Study of Biomimetic Polyene Cyclization

In contrasts the older method of synthesis which uses sequential annulations in a stepwise fashion, **Biomimetic Polyene Cyclization** is a method of synthesis utilizing a single reaction to form several rings with a cation-□ mechanism.

Looking at the biosynthesis pathway of Squalene, Progesterone and other higher Sterol, it will be clear that the key steps of cyclization of linear polyenes involve the following steps (**Figure 3.48**). The general process of cyclase cyclization of linear polyenes:

1) Generation of a carbocation
2) Control of the conformation of the substrate
3) Stabilization of intermediate
4) Quenching of the final carbocation

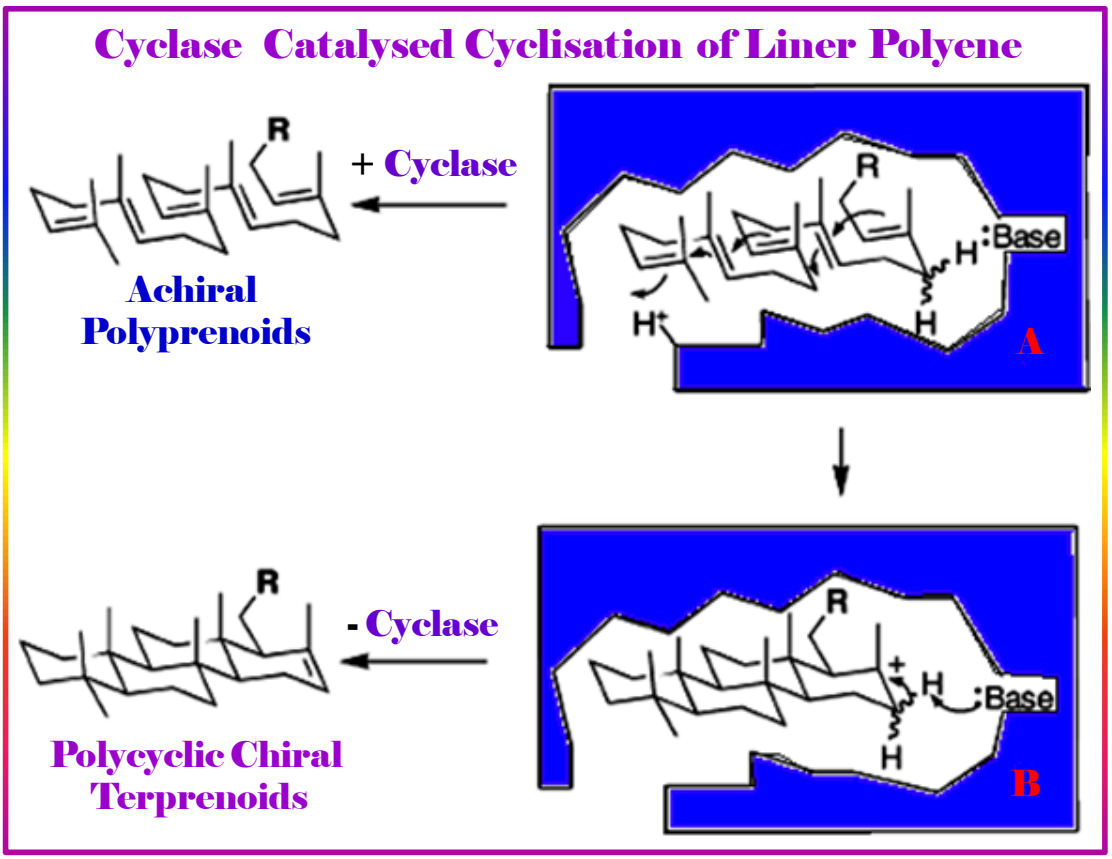


Figure 3.48: The general process of cyclase cyclization of linear polyenes.

The following facts are also further clear from the general process of cyclase cyclization of linear polyenes as well as from the squalene biosynthesis:

- Potential control of stereochemistry. One step produces four rings, 8 stereogenic centers and 1 out of 128 possible isomers.
- Readiness with which highly substituted carbon-carbon bonds may be formed.
- Only *trans* fused rings formed.

Squalene cyclization to lanosterol drew attention to various research groups. And Professor Adolf Windaus in his Nobel Lecture in 1928 rightly stated "The synthesis of such a substrate appears to the chemist particularly difficult, and up till now I have not dared to attempt it".

However, it is the all time desire of chemists to recapitulate nature's activity/productivity and then to mimic it. It is this everlasting desire that drive Professor Sir Robert Robinson to devise the route of biomimetic synthesis of troponone for the first time in the history of biomimetic synthesis who stated that "There has thus been a tendency to explain … that plants have…enormously powerful reagents that are able to cause substances… to undergo transformations which cannot be induced in the laboratory……." And "To a

certain extent…this must be true, but it is probable that this aspect has been exaggerated and that an equally important cause of the variety and complexity of syntheses in plants resides in the highly reactive nature of the substances which function as intermediate products."

Corey et al. have correlated nicely between the function of squalene cyclase enzymes and the goals for biomimetic synthesis as has been stated below (Wendt, K.U.; Schulz, G.E.; Corey, E.J.; Liu, D.R. Angew. Chem. Int. Ed. **2000**, 39, 2812-2833.)

Function of Squalene Cyclase Enzymes	***Goals for Biomimetic Synthesis***
Presents Brønstead acid of sufficient strength	Form carbocation specifically at polyene terminus
Enforces stereochemistry (when non-chair is required)	Control stereochemistry (when non-chair is desired)
Shields cationic intermediates from premature quenching	Prevent premature termination of cascade cyclization
Stabilizes carbocations	Stabilize carbocations
Terminates cyclization	Terminate regioselectively to produce only one isomer

The total synthesis of nature products in the earliest days were made successful by combining the available synthetic methods with a contemporary understanding of biosynthesis. Thus, in those days, biosynthetic considerations are largely inspiration for a synthesis route to a target. When a target's biosynthetic route was unknown, total synthesis played an important role to inspire discrete hypotheses about a target's biosynthesis. However, there is now a clearer understanding of the mutual benefits. The ultimate outcome of mutually exclusive approach is the development of synthetic methodologies that merge upon the efficient techniques of nature. Biosynthetic hypotheses have also guided experiments to establish natural product structure.

In natural enzymatic system, enzymes are able to activate relatively unreactive substrates, such as simple olefins, to undergo multiple bond-forming reactions with high selectivity. In contrast to synthetic systems in which numerous pathways compete with carbocyclization, enzymes exert a miraculous level of control to produce just a few of these possibilities that ultimately lead to high selectivity for the overall transformation. The polyene carbocyclization provides an enzyme model for remarkable multifaceted selectivities in ring size, number of

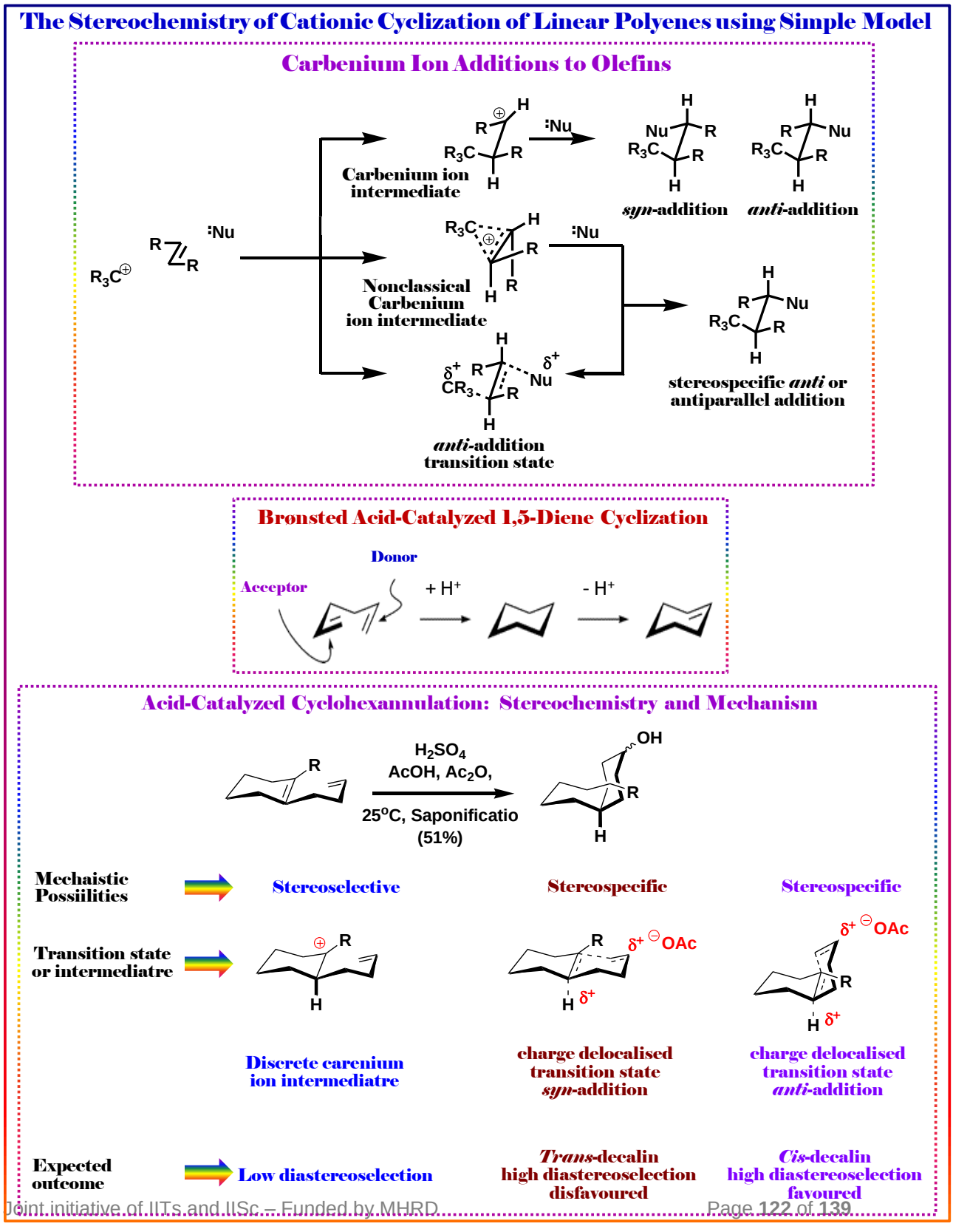
rings formed, stereochemistry of fused ring formation, and the degree of atom/group rearrangement.

Here, I am providing few examples of a case study in polyolefin cyclizations in which the biosynthesis−total synthesis interplay presents an incontrovertible synergism.

The milestone in Biomimetic Cyclization of Linear Polyenes follows:

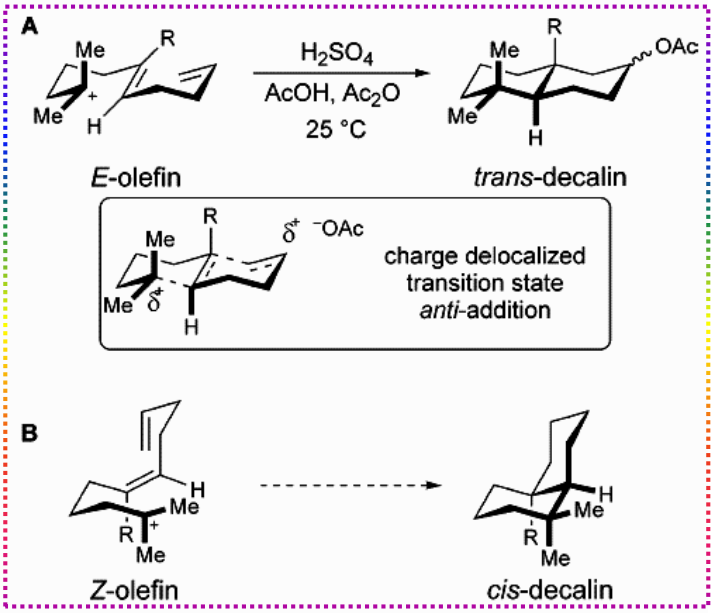
1. **1950s → Study of the stereochemistry of cationic cyclization of linear polyenes using simple model (Contribution from Stork and Eschenmoser)**
2. **1980s → Developing initiator, terminator and cation-stablizing group for cyclization, biomimetic pentacyclization (Contribution from Johnson)**
3. **1990s → Application of chiral LBAs in the enantioselective cyclization of linear polyenes (contribution from Yamanoto)**

3.13.2.5.1. Stork's and Eschenmoser's Study of the Stereochemistry of Cationic Cyclization of Linear Polyenes using Simple Model

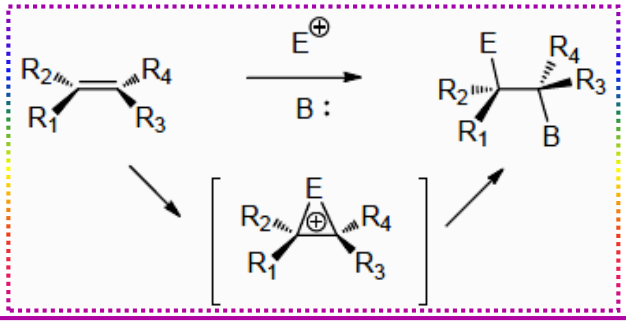


Stork-Eschenmoser Hypothesis

- Polyalkenes react in defined conformations.
- Can predict stereochemistry of cyclization product from starting materials.
  - Z-alkene gives *cis*- ring fusion , *cis*-decalin
  - E-alkene gives *trans*- ring fusion, *trans*-decalin



- Proceed through a concerted mechanism, leading to high Stereospecificity.
- It is a concerted anti addition, the carbenium ion and the olefin donor is anti-arrangement to the olefin aceptor.
- Proceed through a non classical three membered transition state and add in an anti geometry.



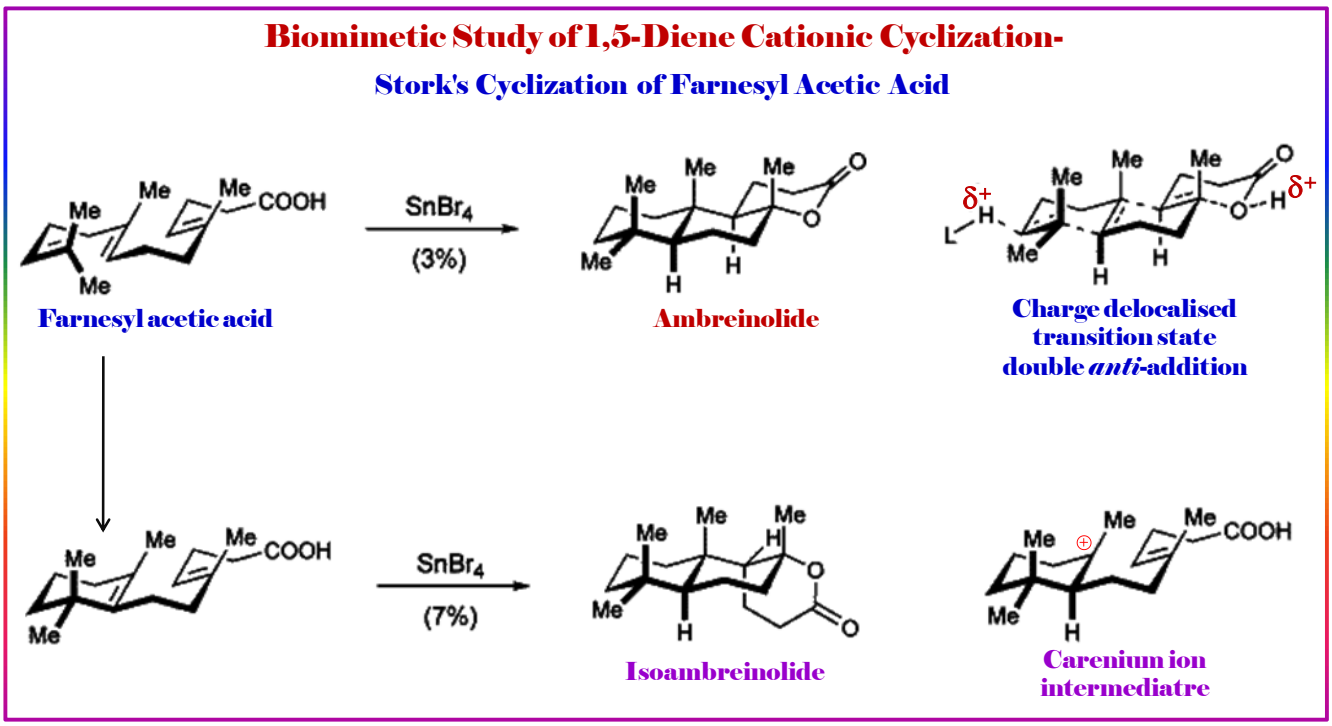


Figure 3.49: Stork's biomimetic cationic cyclization of farnesyl acetic acid to Ambreinolide/Iso ambreinolide.

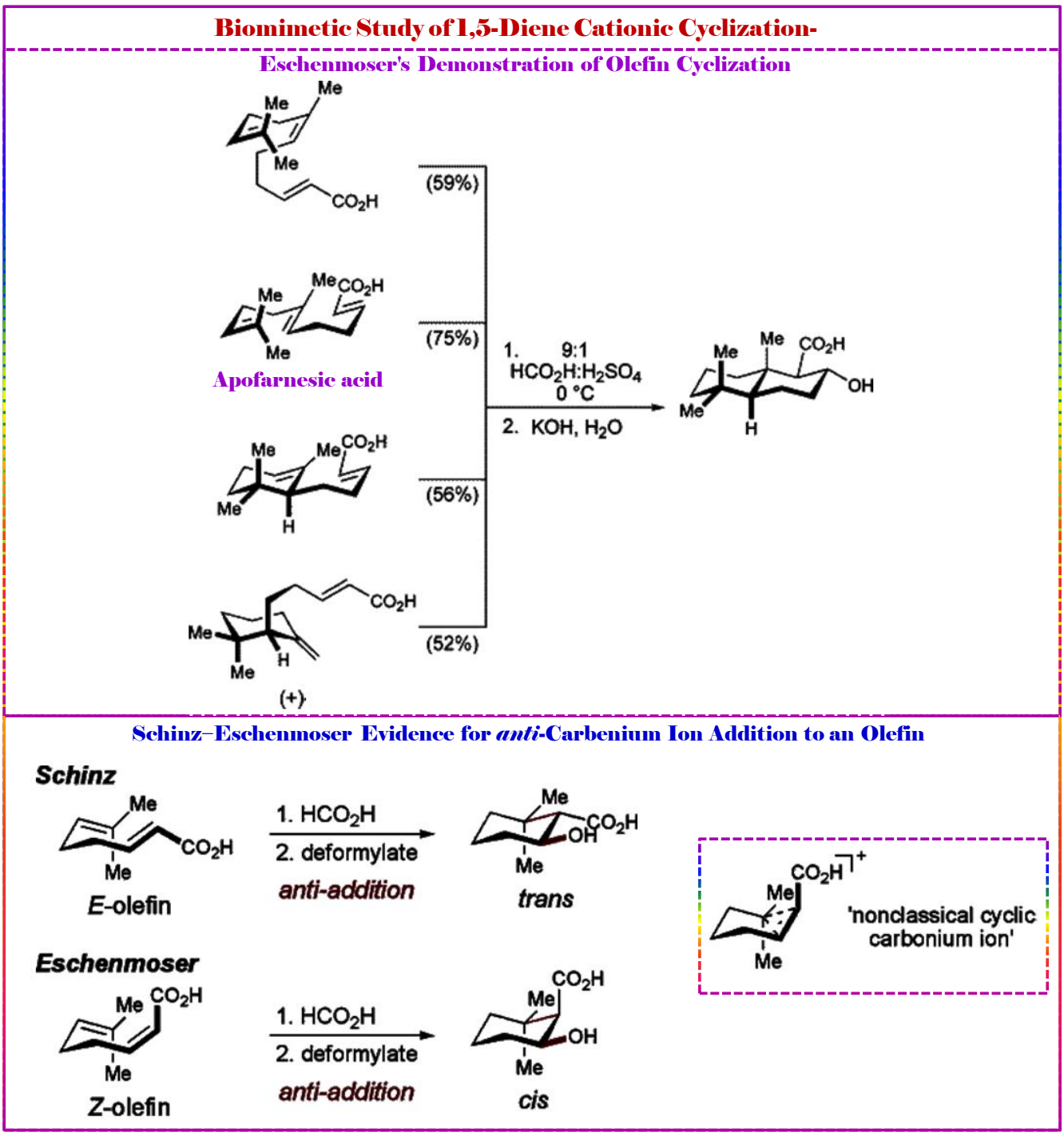


Figure 3.50: Eschenmoser and Schinz-Eschenmoser's biomimetic cationic polyene cyclization.

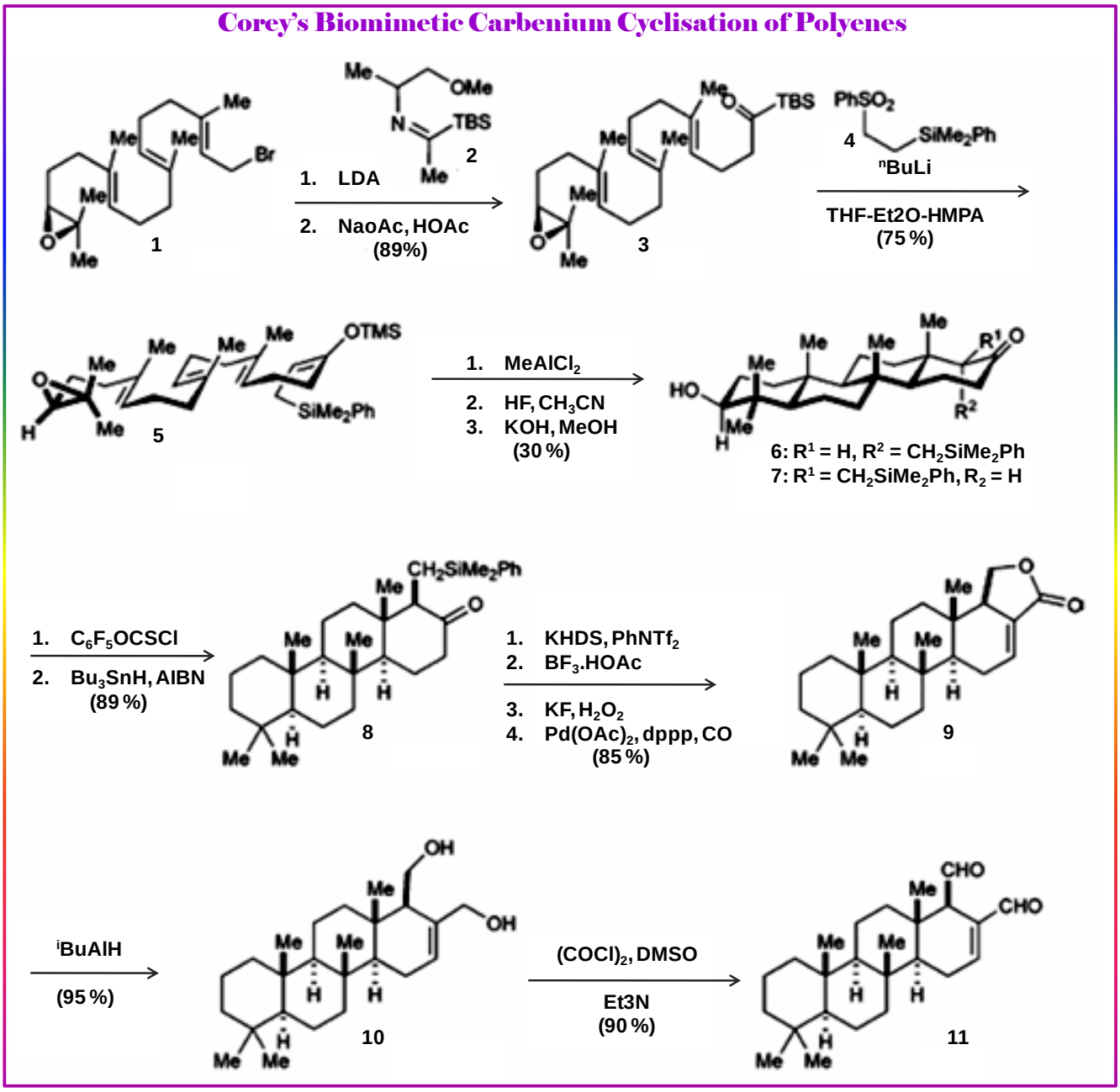


Figure 3.51: Corey's biomimetic cationic polyene cyclization.

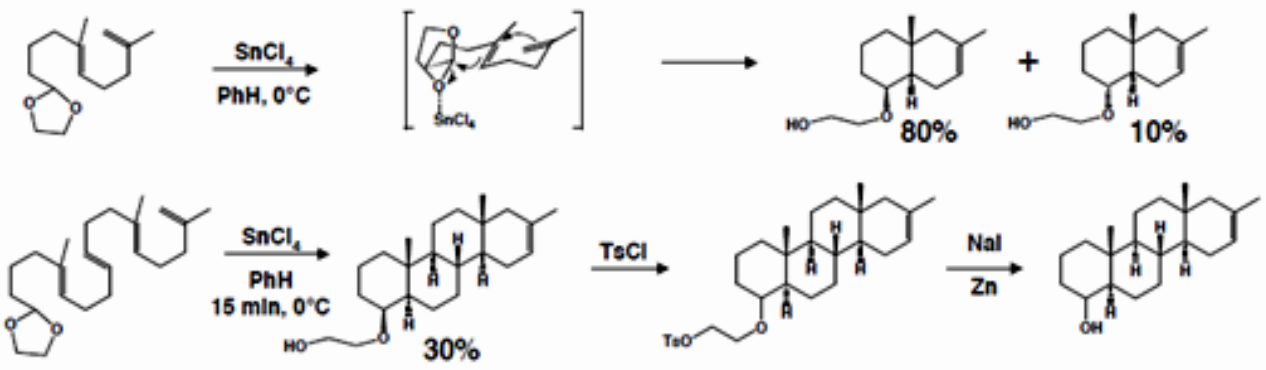
3.13.2.5.2. Johnson's Development of Initiator, Terminator and Cation-Stabilizing Group for Cyclization, and Biomimetic Pentacyclization

Efficient Cyclizations – Initiation of Cyclizations by an Initiator Group

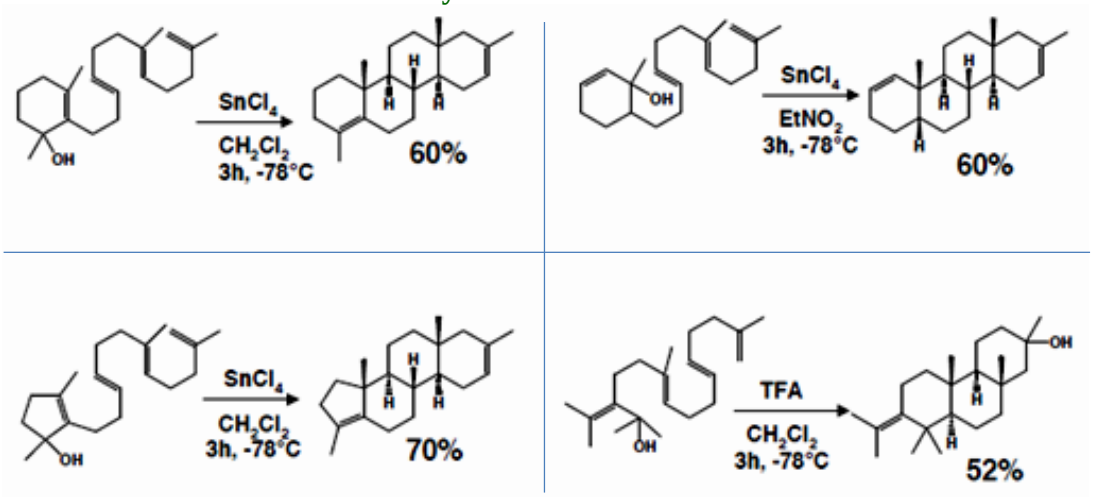
Cyclization Initiation can be achieved

- Through carbocation creation-electrophilic addition or ionization.
- Induced by mild conditions that don't affect olefins or other functional groups.
- Most efficient initiators are stabilized by a heteroatom or double bond.
- Reduced electrophilicity of cation favors a more concerted cyclization-anchimeric effect.
- "Unless an initiator gives nearly quantitative yields in bicyclization, it will not be satisfactory for promoting polycyclizations"--**W.S. Johnson**

Johnson's Chiral Acetal as Initiator



Johnson's Allylic Alcohols as Initiators

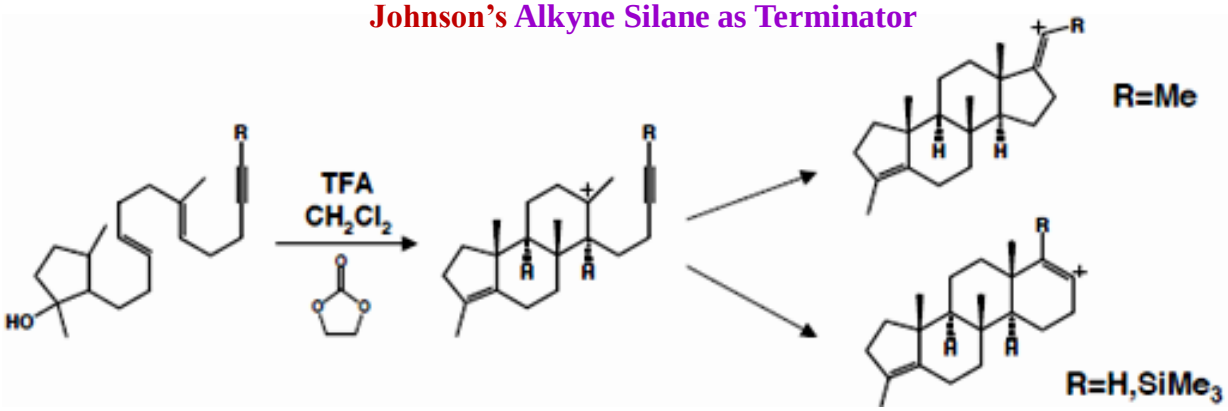


Efficient Cyclizations – Termination of Cyclizations by a Terminator Group

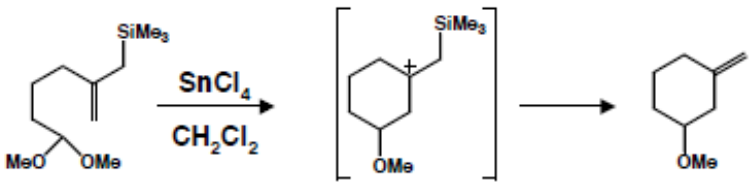
Cyclization Termination can be achieved

- by elimination or attack by nucleophile.
- Termination by one mechanism, giving one product.
- Cyclization Termination is Regioselective.
- Cyclization Termination creates appropriate functionality.
- **Johnson's developed alkyne silane as terminator.**

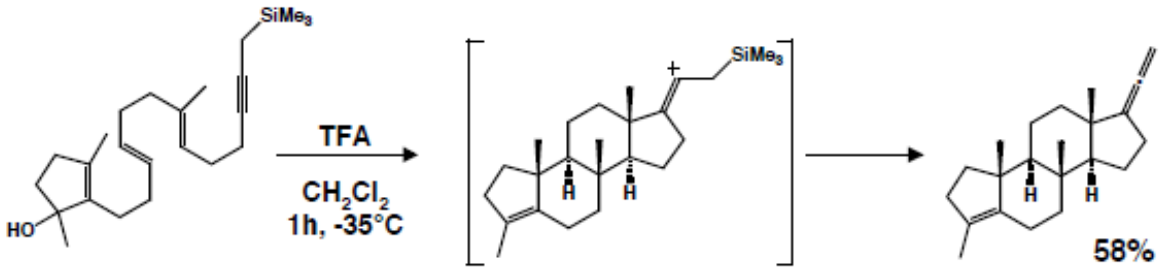
Johnson's Alkyne Silane as Terminator



Johnson's Silane Termination



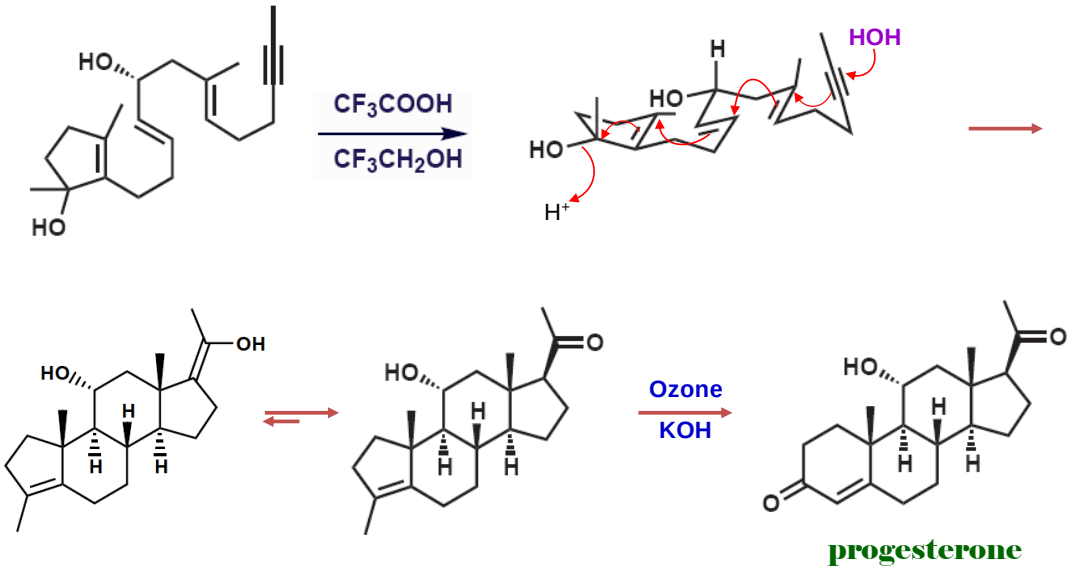
Only product observed



Biomimetic Total Synthesis Based on Squalene Cyclase

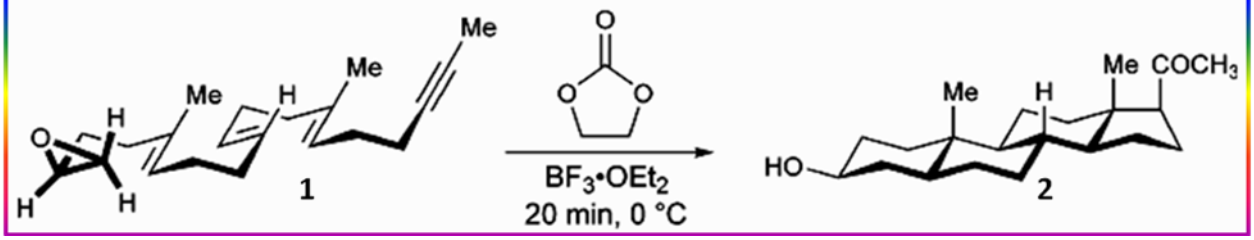
W. S. Johnson's Biomimetic Polyene–Alcohol Cyclization

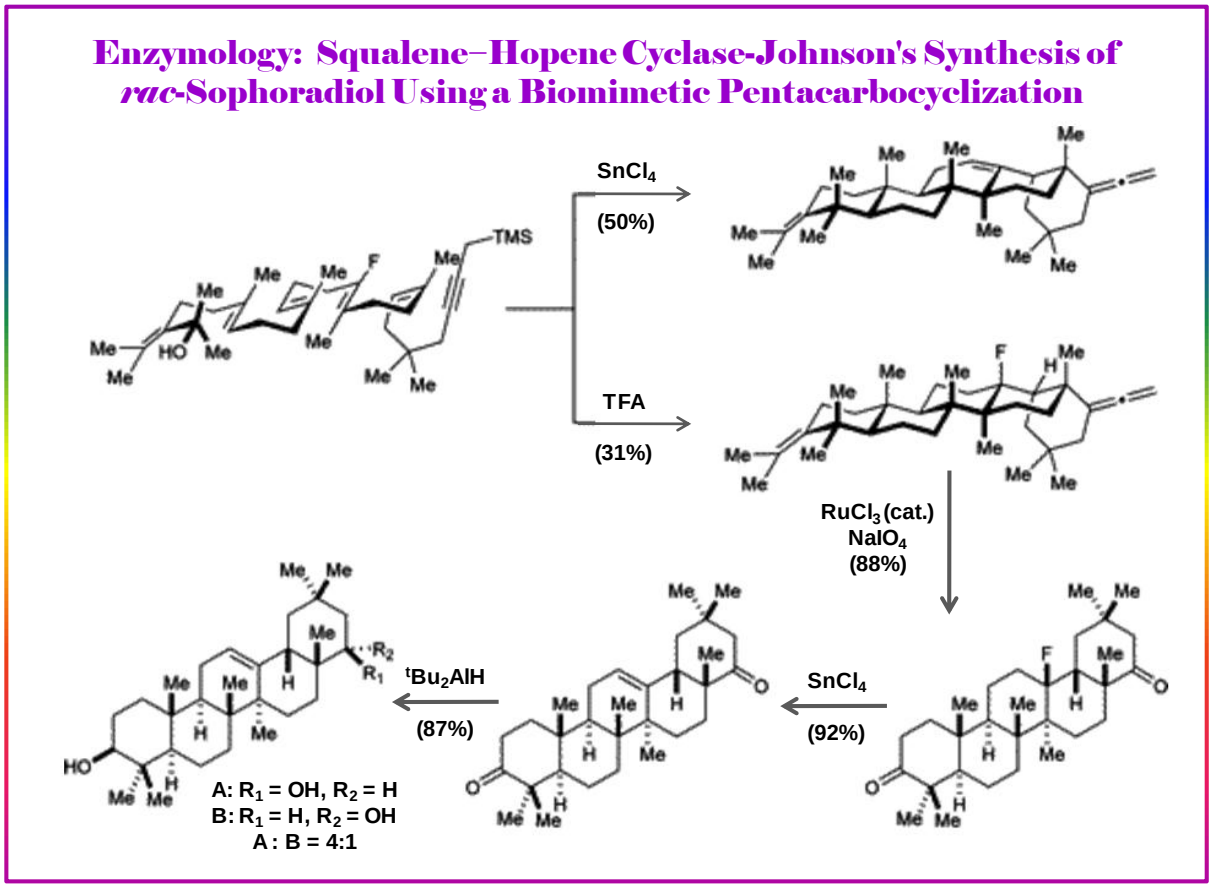
- **Prior to the synthesis of progesterone, triple bonds had not been used in polyene cyclization reactions.**
- **Johnson showed that alkynes could be used to produce exo cyclizations resulting in vinyl cations**



The conversion of 2,3-oxidosqualene to lanosterol is a archetypal cascade reaction in that an epoxide with one stereogenic centre is transformed to a tetracyclic steroid containing seven stereogenic centres. The efficient pioneering synthetic works of van Tamelen and Johnson demonstrated that nonenzymatic, biomimetic total syntheses based on polycarbocyclizations can be achieved easily. In 1961, van Tamelen used the term biomimetic, or biogenetic-type, as "an organic synthesis designed to follow, in at least its major aspects, biosynthetic pathways proved, or presumed, to be used in the natural construction of the end product." Early work in the epoxysqualene biomimetic carbocyclization proved difficult due to the necessary formation of a six-membered C-ring closure over a favoured 5-*exo* Markovnikov addition. However, in 1982, van Tamelen utilized an alkyne as the terminating group in his cascade cyclization (**1**), which allowed for formation of both the six-membered C-ring and the desired five-membered D-ring (**2**) (below example).

van Tamelen's Alkyne Termination Strategy to Biomimetic Cyclisation





3.13.2.5.3. Application of Yamamoto's Chiral LBAs in the Enantioselective Polyenes Cyclization

Yamamoto developed the first enantioselective biomimetic cyclization of a polyprenoid using a Lewis acid-assisted chiral Brønsted acid, LBA in 1999 (**Figure 3.52**) as an *artificial cyclase* and used it to synthesize (−)-ambrox (**4**). Ambrox is used as a commericial substitute for ambergris and is found in such fragrances as Givenchy's Extravagance d'Amarige, due to its unique olfactory and fixative properties. The cyclization of homofarnesol (**3**) promoted by LBA **1** proceeded with 42% ee (**Figure 3.53**). This enantioselective cyclization was further improved in 2002 using LBA **2** as the promoter. The ether (−)-**4** was obtained in 54% yield with 75% ee and 76% dr from **5** utilizing an enantioselective cyclization, silylation, and diastereoselective cyclization sequence (**Figure 3.53**). Thus, the best results were achieved by use of an achiral Lewis acid in the subsequent silyl ether cyclization.

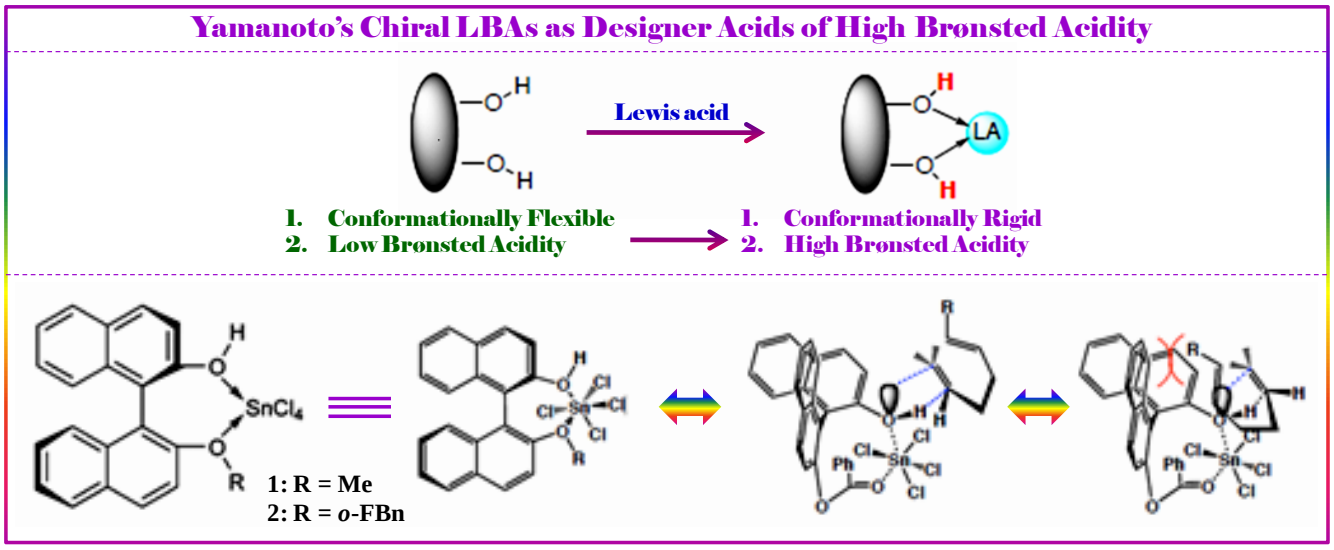


Figure 3.52: Yamamoto's concept of designing Chiral Lewis-*Brønsted Acid*, LBA and its structure.

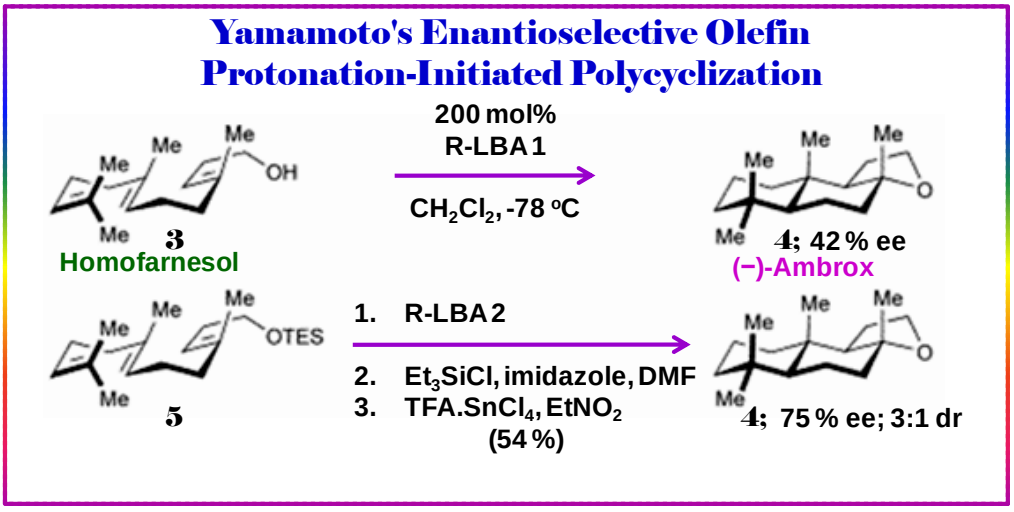


Figure 3.53: Yamamoto's biomimetic synthesis of (−)-ambrox using LBA.

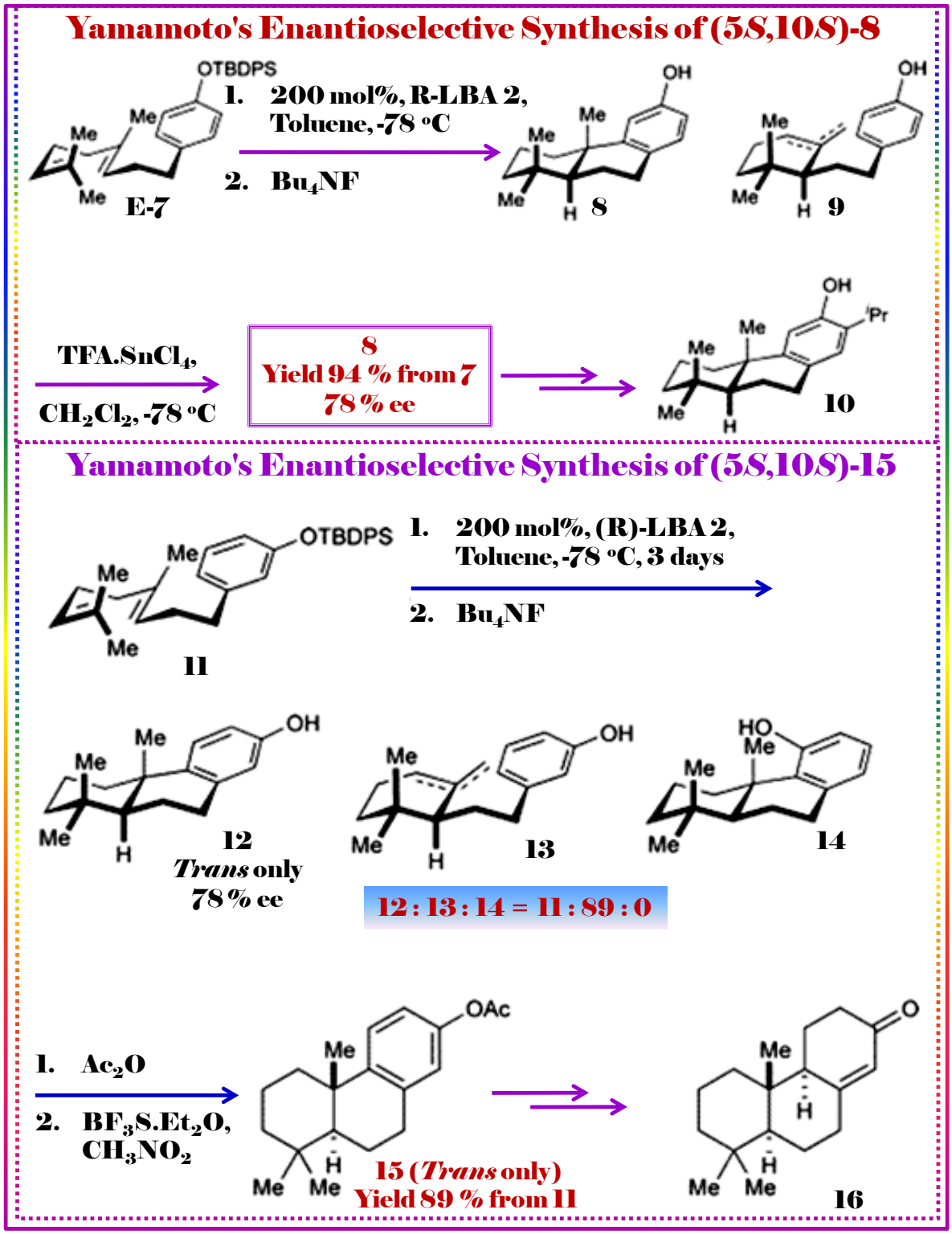


Figure 3.54: Yamamoto's biomimetic polyene cyclizations using LBA.

3.13.2.5.4. Polyenes Cyclization Based on Radical Transformations

The speculation of Breslow in 1962 was made possible to explore radical mediated synthesis of sterol from squalene. He thought that the enzyme may exert conformational control to promote the radical cyclization in the biosynthetic pathway of sterols from squalene though squalene would not cyclize in the presence of hydroxyl radical. However, the radical pathway has not been exploited successfully in several polycarbocyclizations. Breslow clearly stated in his model study their findings may be of limited interest, but it became the foundation for the remarkable transformations and establish that carbenium ions are not *necessary* intermediates for diastereoselective carbocyclizations in the stereocontrolled formation of terpene frameworks.

Breslow in his model study used a CuCl-catalyzed thermal decomposition to benzoyloxy radicals with cupric benzoate as an added terminator. This protocol afforded the cyclized product in 20-30% yield (**Figure 3.55A**). The relative stereochemistry of **2** reflects that of □-onocerin (**3**), a product of the enzymatic cyclization of squalene.

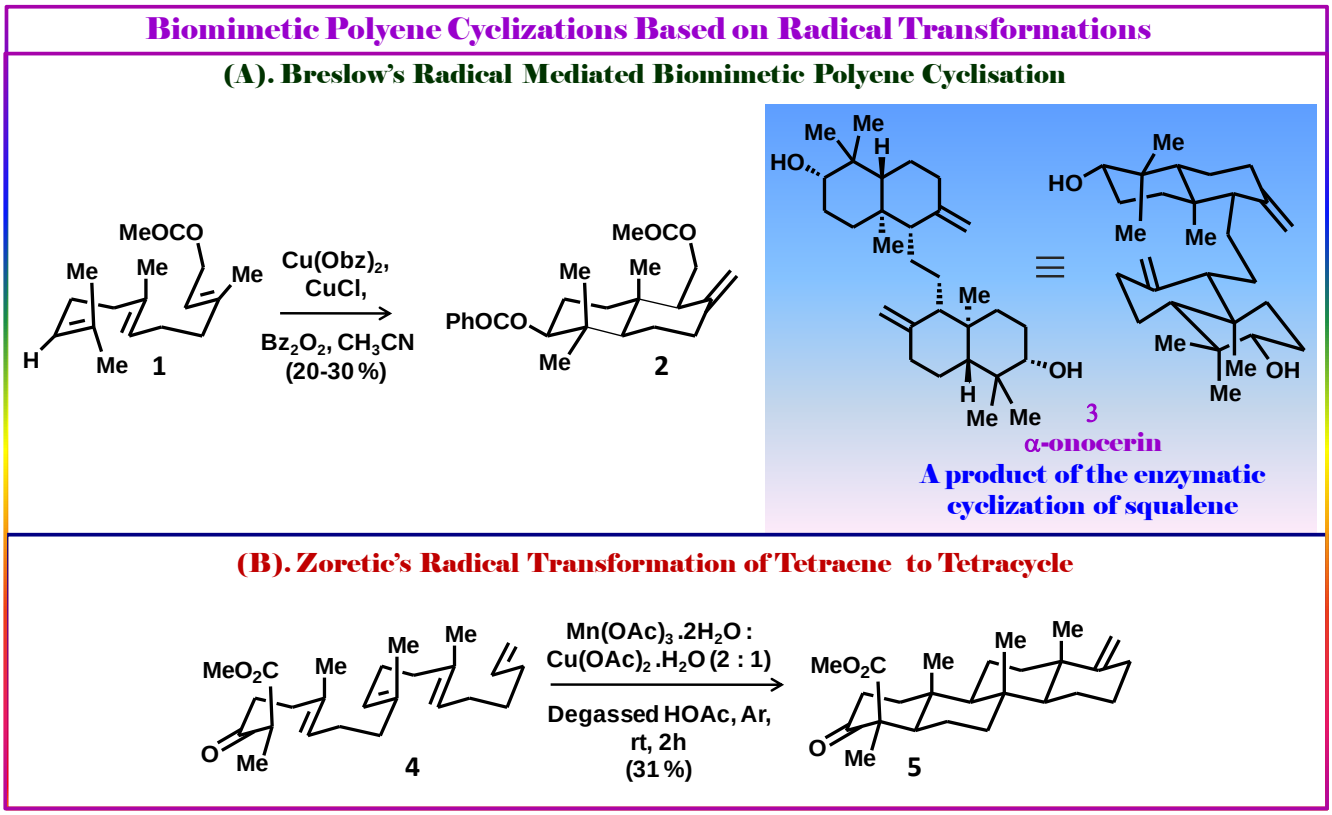


Figure 3.55: Biomimetic polyene cyclizations based on radical transformations-Contribution by (A) Breslow and by (B) Zoretic.

Snider developed manganese(III)-based oxidative free-radical cyclizations in the presence of copper(II) in 1990. Snider's work led to the formation of a wide variety of fused ring systems. Later on, in 1991, Zoretic was able to transform tetraene **4** to tetracycle **5** in 31% yield using the Mn(III)/Cu(II) combination (**Figure 3.55B**) wherein seven stereogenic centers are formed in an apparent diastereoselective reaction.

This above homolytic polycyclization is not limited to radical cation intermediates. This was proved by Pattenden by generating an acyl radical from an acyl selenide and $Bu_3SnH-AIBN$ which ultimately led to a tetracyclisation to occur (**Figure 3.56A**). This transformation creates six new stereogenic centers with a high level of diastereoselection.

This strategy was more recently elaborated by Demuth to a short biomimetic synthesis of a steroidal skeleton. (−)-Menthone was used by Demuth as a remote chiral auxiliary on a terminus of the polyalkene (**8**). Photoinduced electron transfer then initiated the polycyclization by formation of radical cation **10**. From the resulting cascade, eight stereogenic centers were created to form only two of the greater than 200 possible isomers (**Figure 3.56B**). The resulting ketal **9** is formed in 10% yield as a 7:1
diastereomeric ratio. Separation of the diastereomers and removal of the chiral auxiliary afforded ester **11**, in >99% ee. This example is a remarkable demonstration of **Remote Asymmetric Induction**. Most importantly, the C3-hydroxy results from water in this approach and hence it provides a possible alternative biosynthetic pathway to produce steroids from epoxysqualene under non-oxidative conditions.

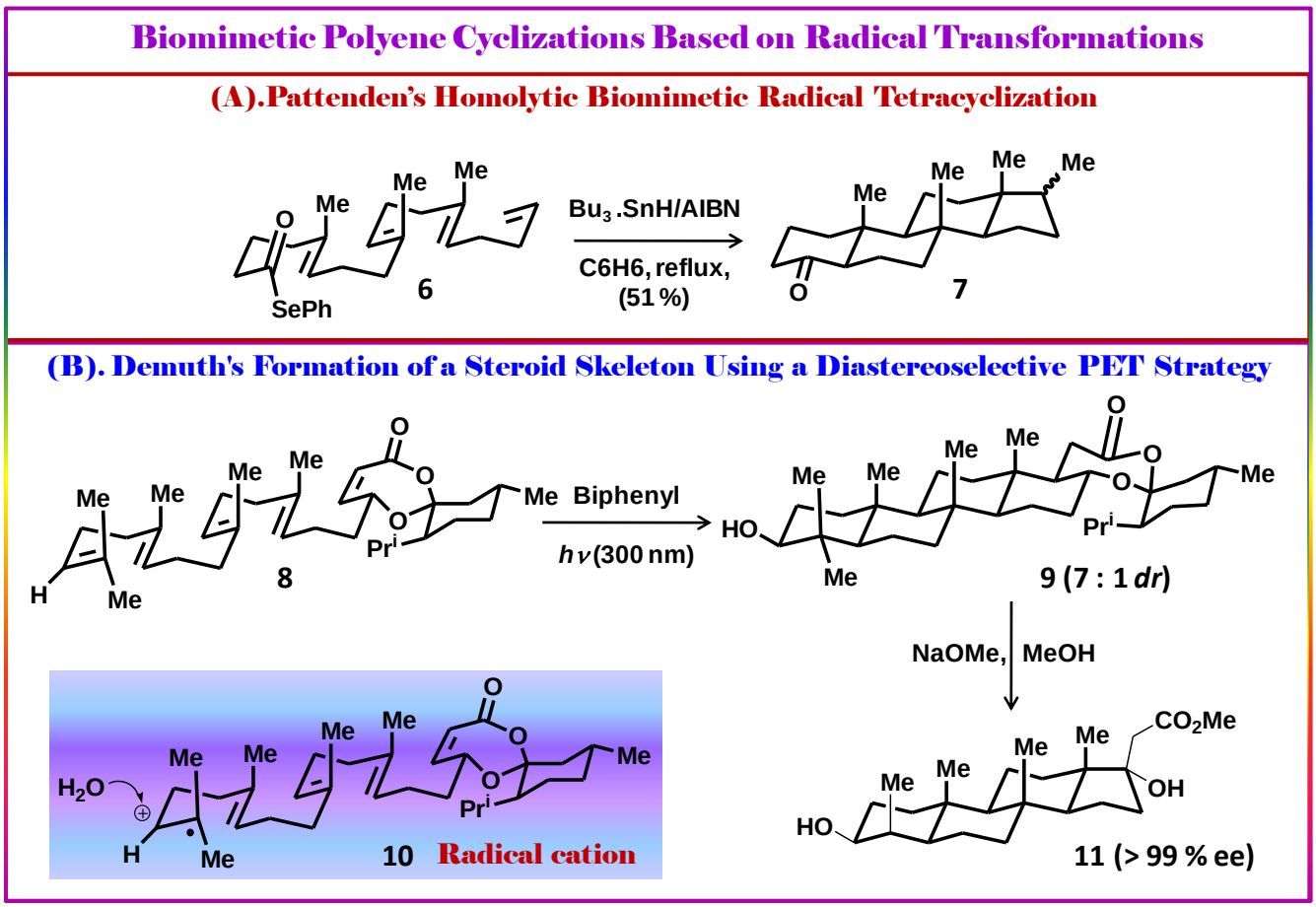


Figure 3.56: Biomimetic polyene cyclizations based on radical transformations-Contribution by (A) Pattenden and by (B) Demuth.

3.13.2.6. Summary and Outlook of Biomimetic Polyene Synthesis

From the above examples and many others it is clear that a lot of efforts have been put forth for the biosynthesis of natural terpenoides utilizing biomimetic polyene carbocyclization. It is also noticeable that in all cases, biosynthesis inspired a key polycyclization that generated substantial complexity in a single transformation. Similarly, from the synthetic studies of the polycyclization enables to discover how to stabilize developing charge at specific carbons in the squalene backbone during cyclization. This same discovery was unfolded much later during mutagenesis studies of cyclase. Therefore, the organic synthesis and biosynthetic studies are evolved concomitantly.

It was thought and considered that selectivity in catalysis is directly related with the complexity of enzyme catalyst (the protein environment) that selectivity may not be attainable with a simple small molecule catalyst. As for example, squalene−hopene cyclase enzyme uses a set of primary and secondary residues to orient and to activate squalene for cyclization. However, discovery of simple small Lewis acid catalyst, LBA by Yamamoto catalysts clearly shows that without functional/conformational complexity a simple catalyst can lead to polyene

cyclization to furnish polycyclic products with high selectivity. Further research is needed to develop biomimetic catalyst wherein both selectivity and *efficiency* can be controlled simultaneously to reach the exact proficiency of a natural enzyme catalyst. Similarly, biomimetic total synthesis will be benefited from biosynthetic thinking and studies. Thus, both are synergistic in their future flourishment.

The value of mimicking biology should be evaluated in a case-by-case manner, yet many successes are achieved in biomimetic total syntheses. In this regard van Tamelen rightly stated: “It seems hardly necessary to add that the success of a ‘biogenetic-type' synthesis by itself does not constitute evidence for the operation of a particular chemical step in nature (although in a remarkable case, the temptation to draw such a conclusion will be great)”.

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3.15. Assignments

1. Is there a difference between the initial and the final energy levels in catalyzed and non-catalyzed reactions?
2. What are the main theoretical models that try to explain the formation of the enzyme-substrate complex?
3. What is the chemical basis of enzyme specificity?
4. What is the Michaelis-Menten equation and its Lineweaver-Burk form?
5. How does the Michaelis-Menten equation explain why the rate of an enzyme-catalyzed reaction is proportional to the amount of enzyme? How does the Michaelis-Menten equation explain why the rate of an enzyme-catalyzed reaction reaches a maximum value at high substrate?
6. How does the formation of an E.S complex explain the reaching of a maximal velocity in the Vo *vs* So graph?
7. What is antibody and what is hapten?
8. Design a hapten for eliciting antibody usable for catalyzing Oxy-Cope rearrangement?
9. The antibiotic penicillin is a small molecule that does not induce antibody formation. However, penicillin binds to serum proteins and forms a complex that in some people induces antibody formation resulting in an allergic reaction. What role then Penicillin serves- an antigen/ a hapten/ an immunogen/ or both an antigen and a hapten?
10. Define the following terms: (a) Enzyme model and (b) Biomimetic Synthesis.
11. Is there a difference between the initial and the final energy levels in catalyzed and non-catalyzed reactions?
12. What are the main theoretical models that try to explain the formation of the enzyme-substrate complex?
13. What is the chemical basis of enzyme specificity?