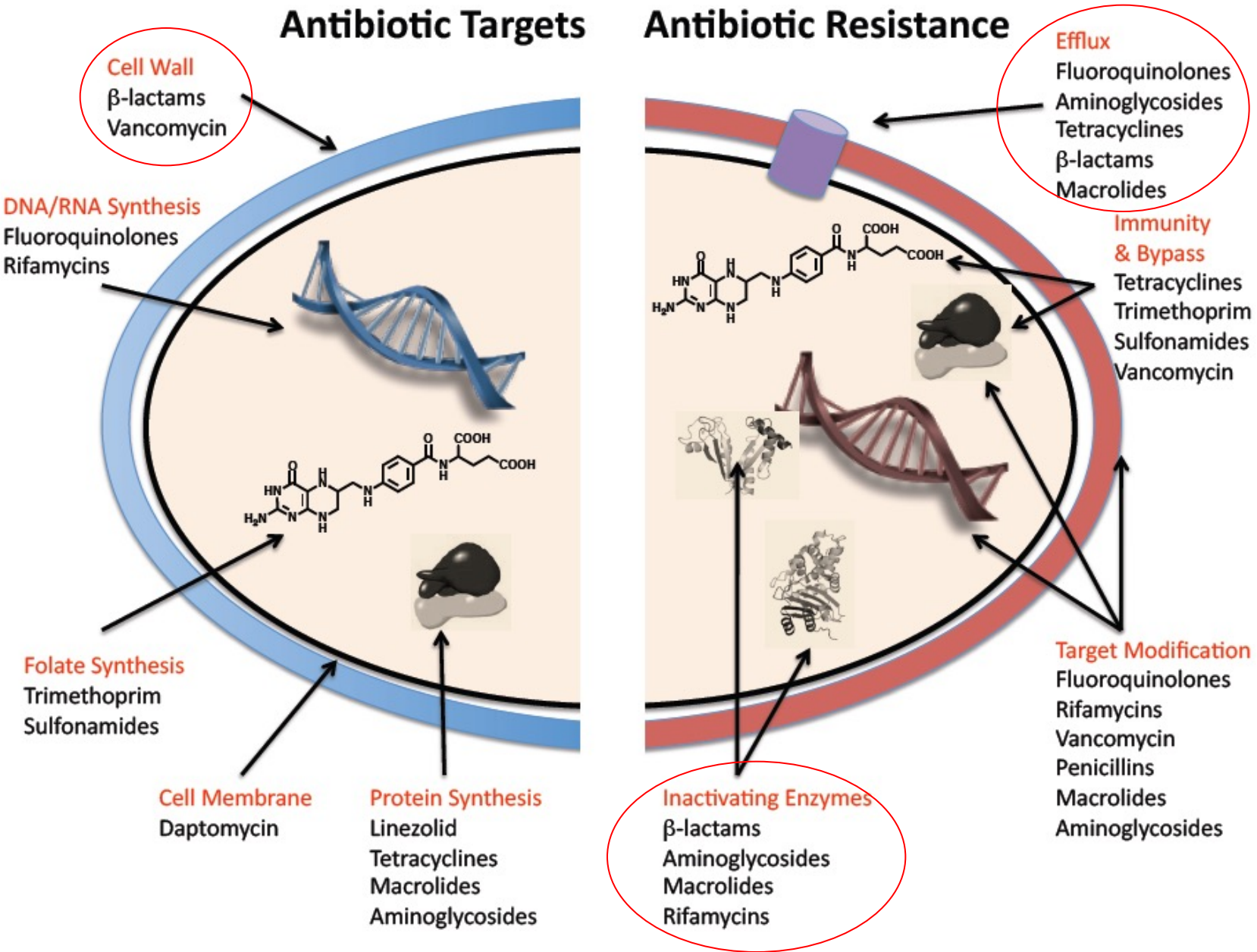
Biosynthesis and modification of β-lactam antibiotics.

Penicillins and cephalosporins

Antibiotic targets and resistance mechanisms



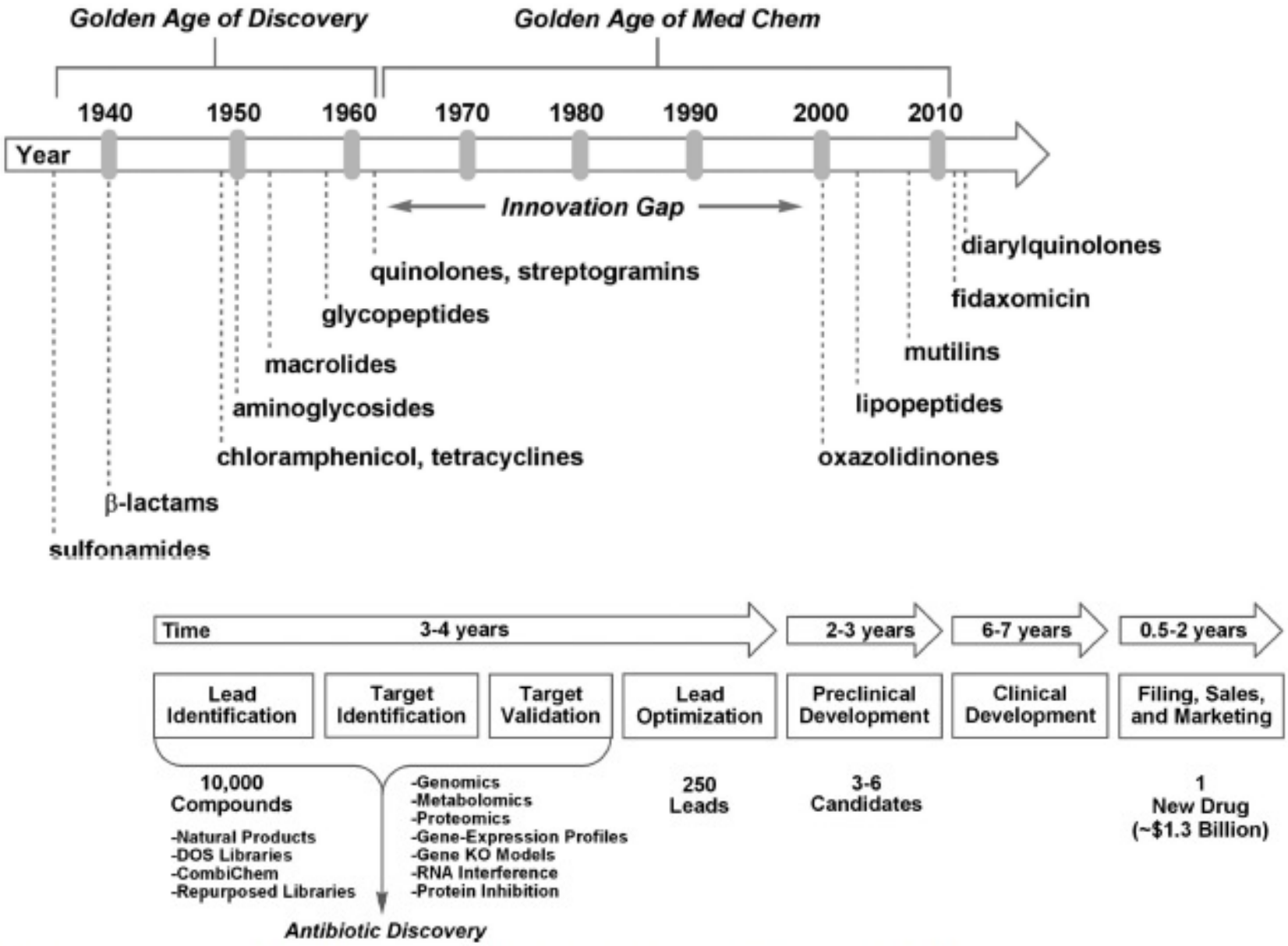
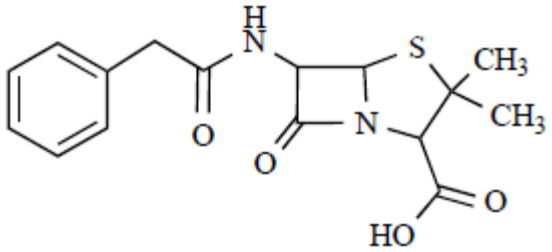


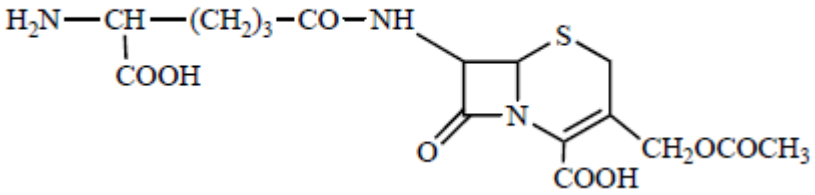
Figure 1 Antibiotic drug discovery and development flow chart adapted from literature versions.[68]

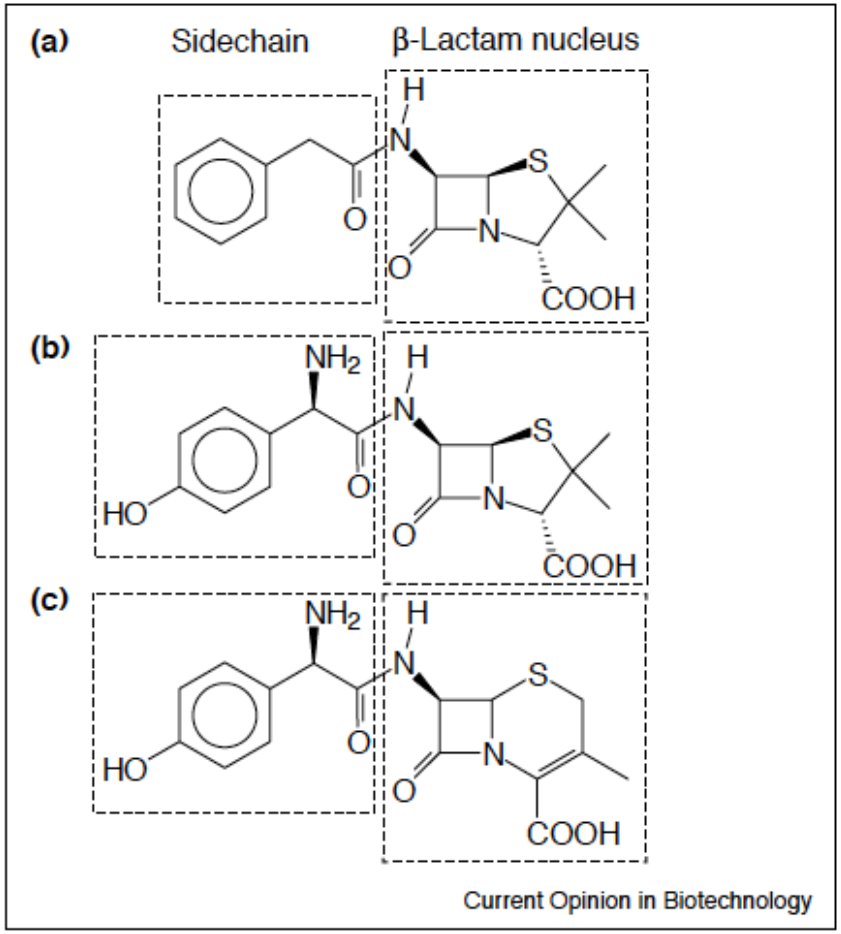
Structures of some β-lactam antibiotics



Penicillin G

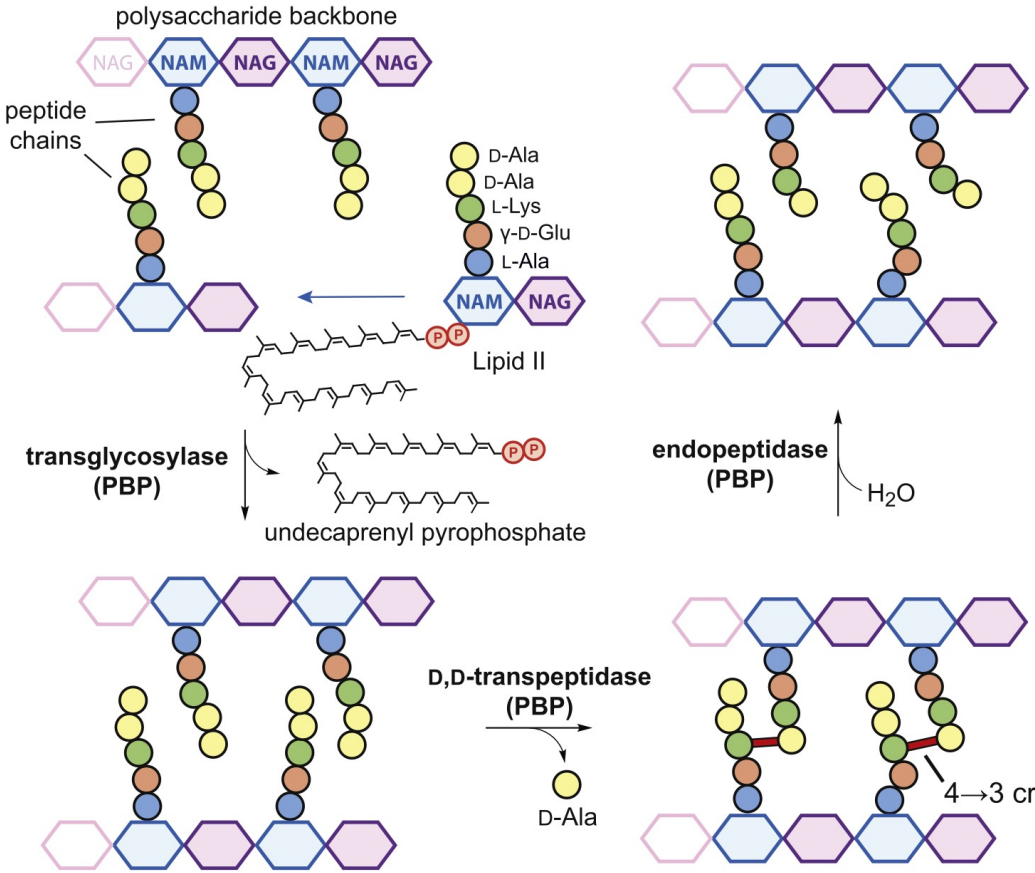
Cephalosporin C

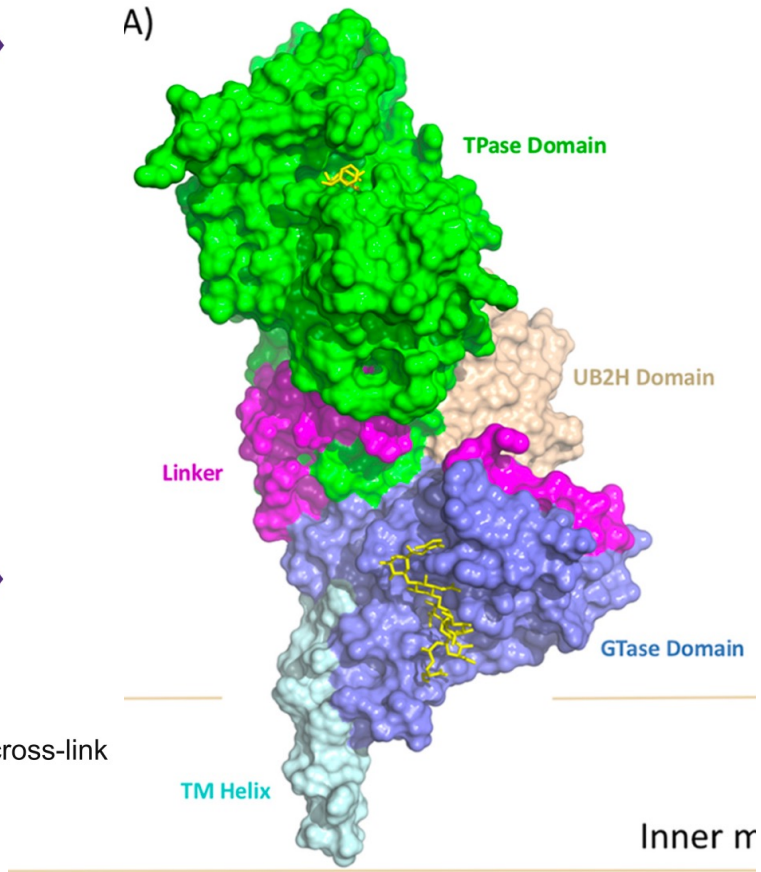




β-Lactam antibiotics comprise a β-lactam nucleus coupled to a sidechain. Examples of two penicillins **(a)** penicillin G and **(b)** amoxicillin and **(c)** a cephalosporin, cephadroxil.

Penicillin-binding proteins (PBP): bifunctional enzymes essential for bacterial cell wall peptidoglycan biosynthesis





Mechanism of action of penicillin and cephalosporin

Penicillin and cephalosporin inhibit transpeptidase, the enzyme responsible for formation of cross-links during the synthesis of peptidoglycan of the bacterial cell wall.

Penicillin and cephalosporin are structural analogues of the natural substrate of transpeptidase.

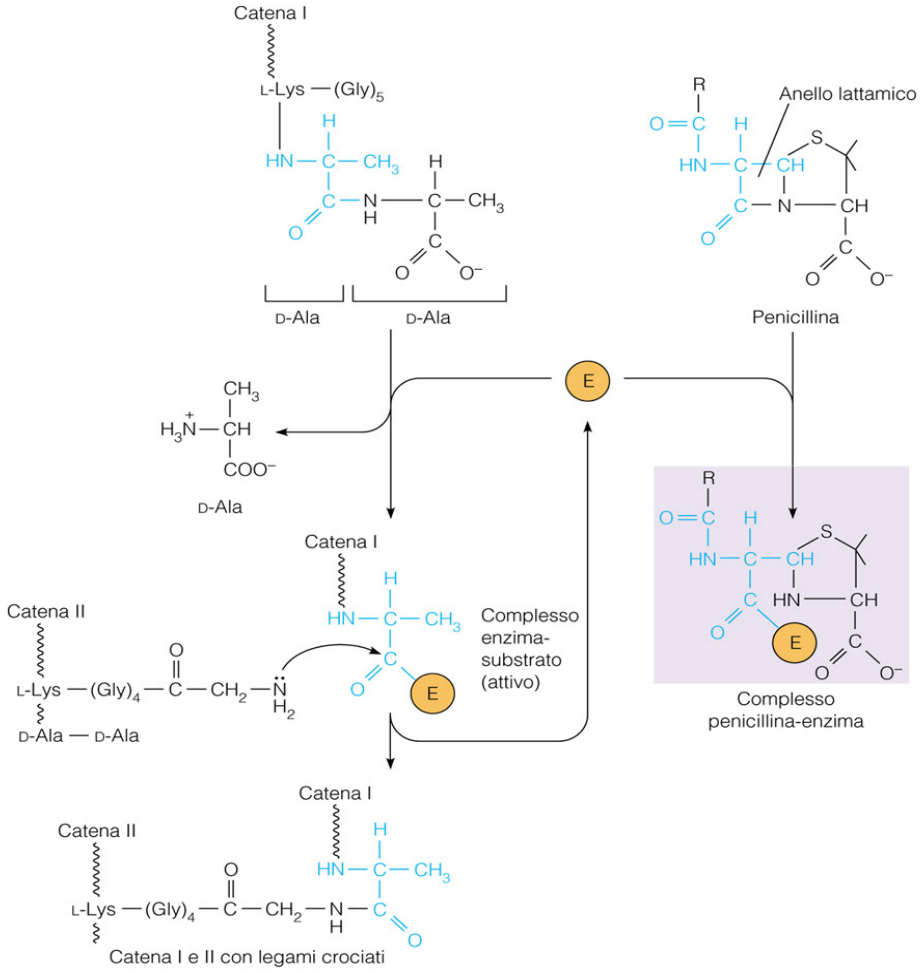
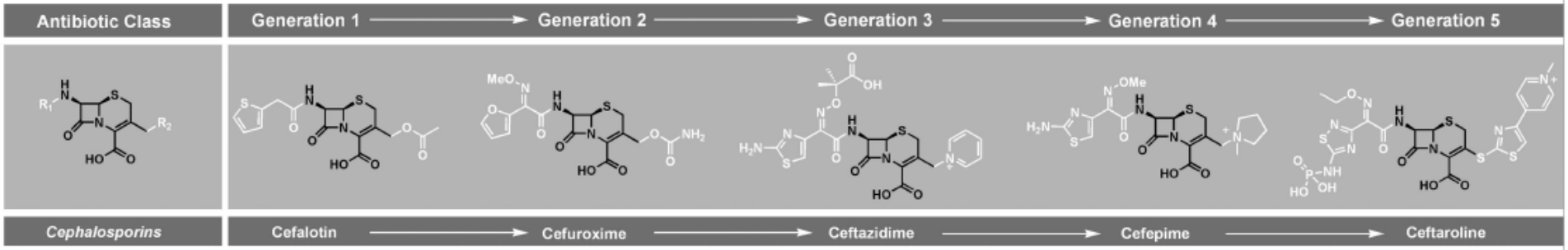


Table 1 Marketed and experimental β-lactam antibiotics. Antibiotics italicized are major commercial antibiotics

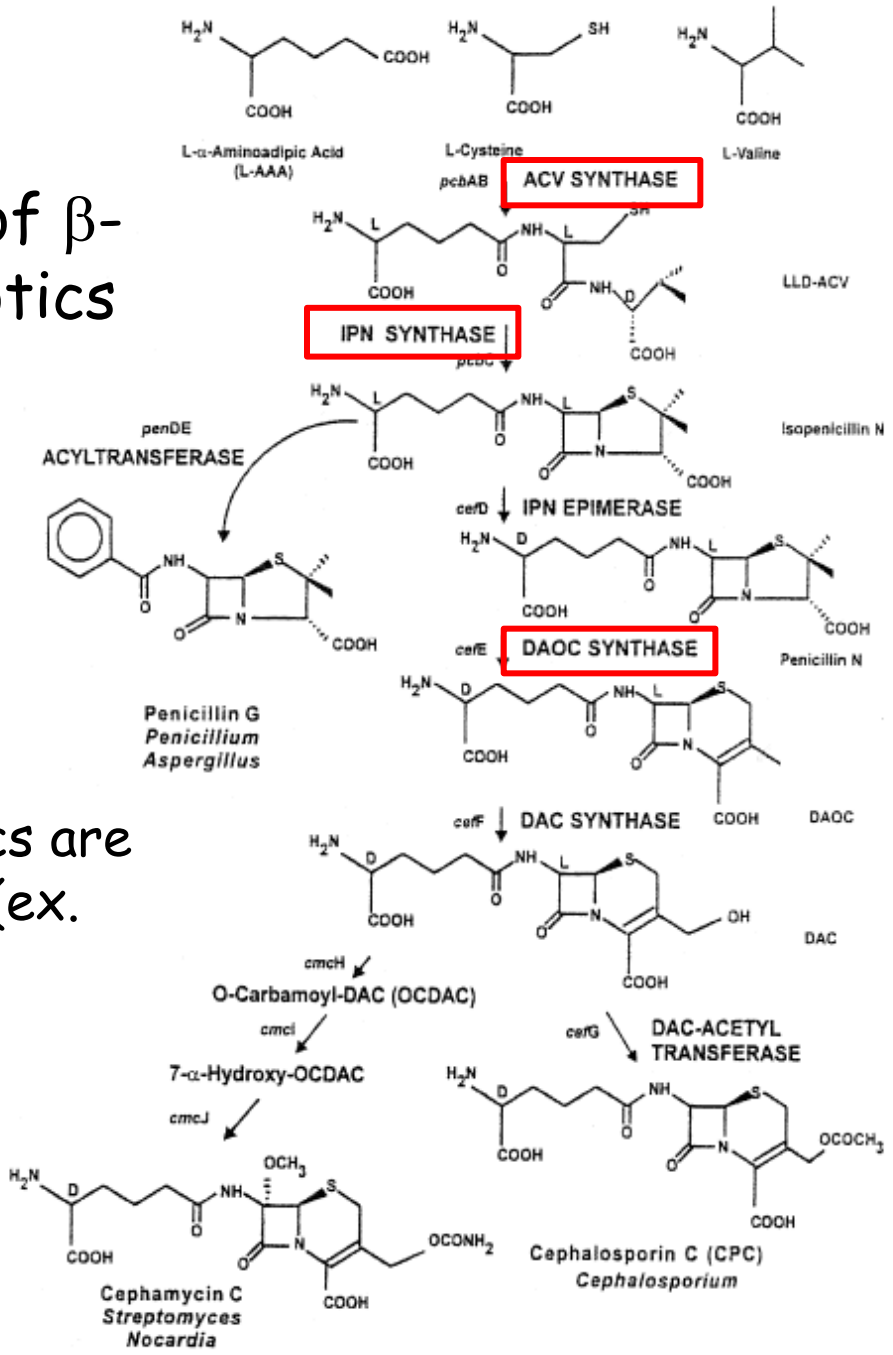
Subclass	Marketed β-lactam antibiotics
Penicillins	Ampicillin[a], *amoxicillin*[a], bacampicillin, cloxacillin, floxacillin, mezlocillin, nafcillin, oxacillin, penicillin G[a], penicillin V[a]
Penicillin-resistant penicillins	*Methicillin*, dicloxacillin
Antipseudomonal penicillins	Carbenicillin, indanyl piperacillin, *ticarcillin*
First-generation cephalosporins	Cefalothin, cephradine[a], cefadroxyl[a], cefazolin, *cephalexin*[a]
Second-generation cephalosporins	*Cefuroxine*, *cefaclor*[a], cefotetam, cefmetazole, cefonicid
Third-generation cephalosporins	*Cefixime*[a], *ceftibuten*, cefizoxime, ceftriaxone, cefamandol cefoperazone, cefotaxime, proxetil, *cefprozil*[a], ceftazidime, cefuroxime axetil, cefpodexime
Fourth-generation cephalosporins	*Cefepime*
Oxycephams	Flomoxef, latamoxef
Cefam	Cefoxitin
Carbapenems	Loracarbef[a], *imipenem*, meropenem, panipenem
Monobactams	Aztreonam, carumonam
Clavams (β-lactamase-inhibitors)	*Clavulanate*, sulbactam, tazobactam
Penicillins/β-lactamase inhibitors	*Amoxicillin/clavulanate*, ampicillin/sulbactam, pipericillin/tazobactam, ticarcillin/clavulanate, cefoperazone/sulbactam

[a] Orally administered β-lactams



Biosynthesis of β-lactam antibiotics

β-lactam antibiotics are produced in fungi (ex. *Penicillium* sp. and *Acremonium* sp.).

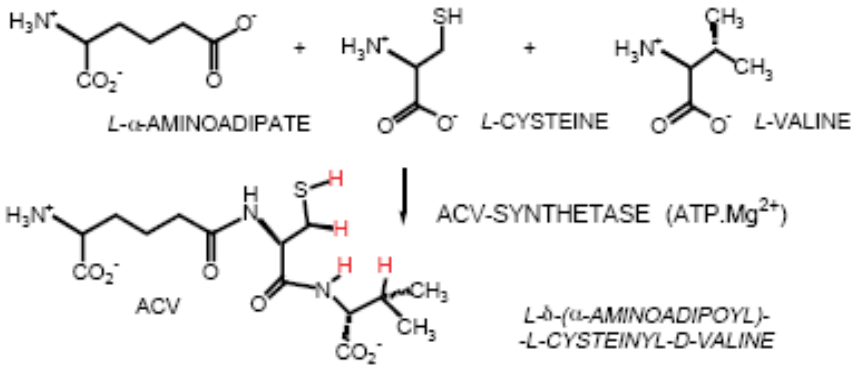


Modular organization of ACV NRPS

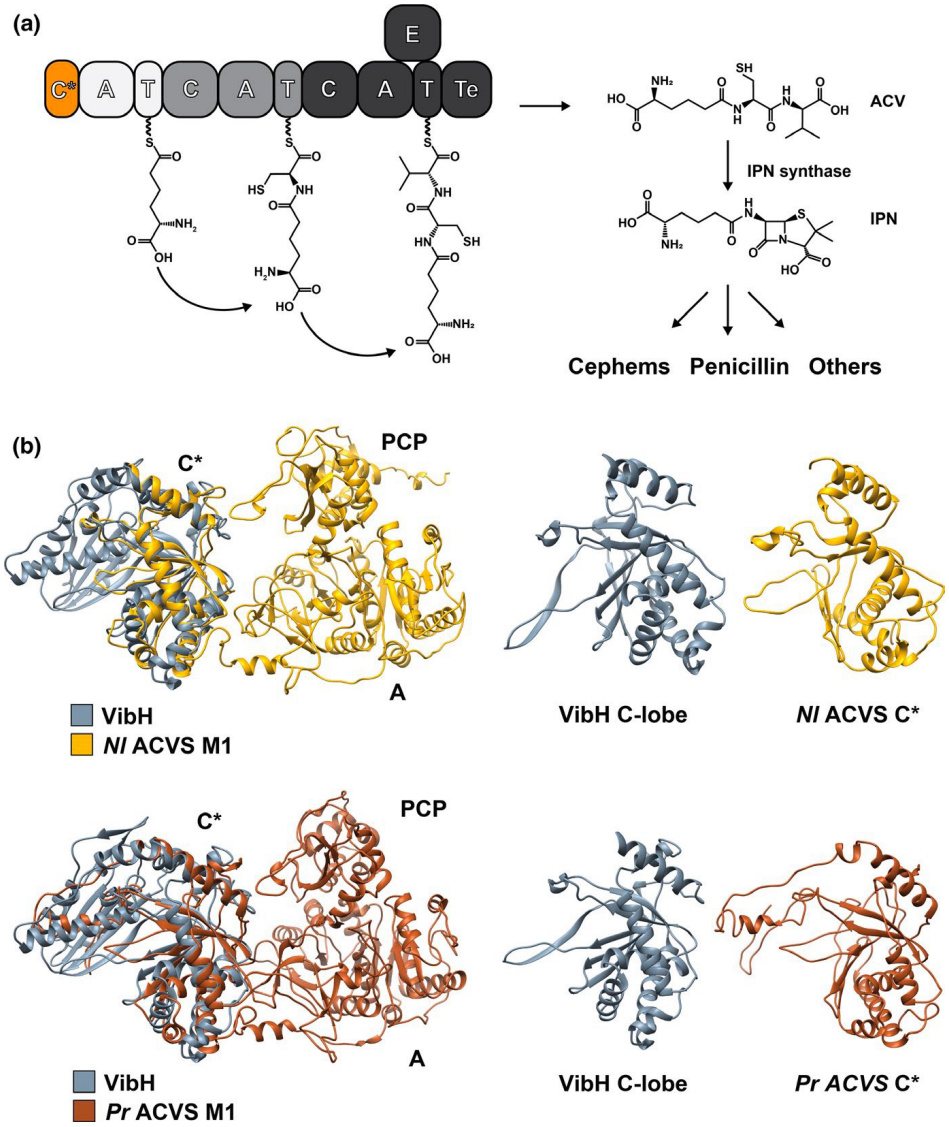
The β-lactam ring precursor is the tripeptide L-δ-α-aminoadipyl-L-cysteinyl-D-valine produced by ACV synthetase.

Aad Cys Val

A-T-C-A-T-C-A-T-E-Te



The first module of ACV NRPS

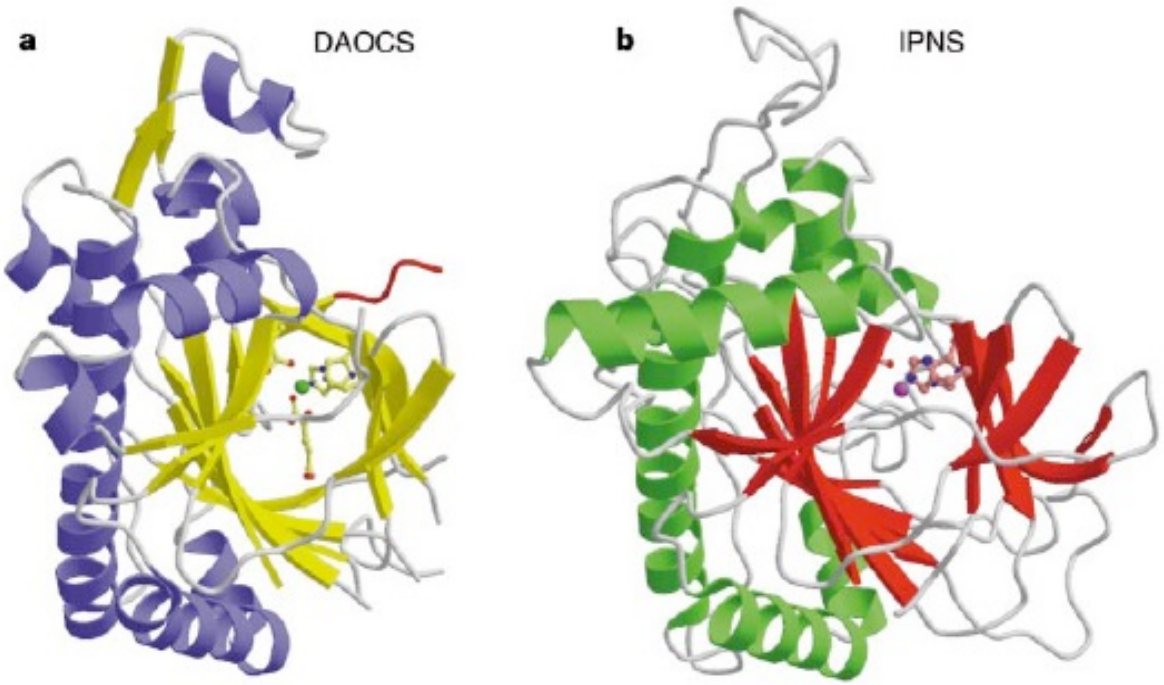


Bionformatic analyses identify an atypical N-terminal domain related to the C-lobe of condensation domains

Deletion of this domain abolishes ACV synthesis

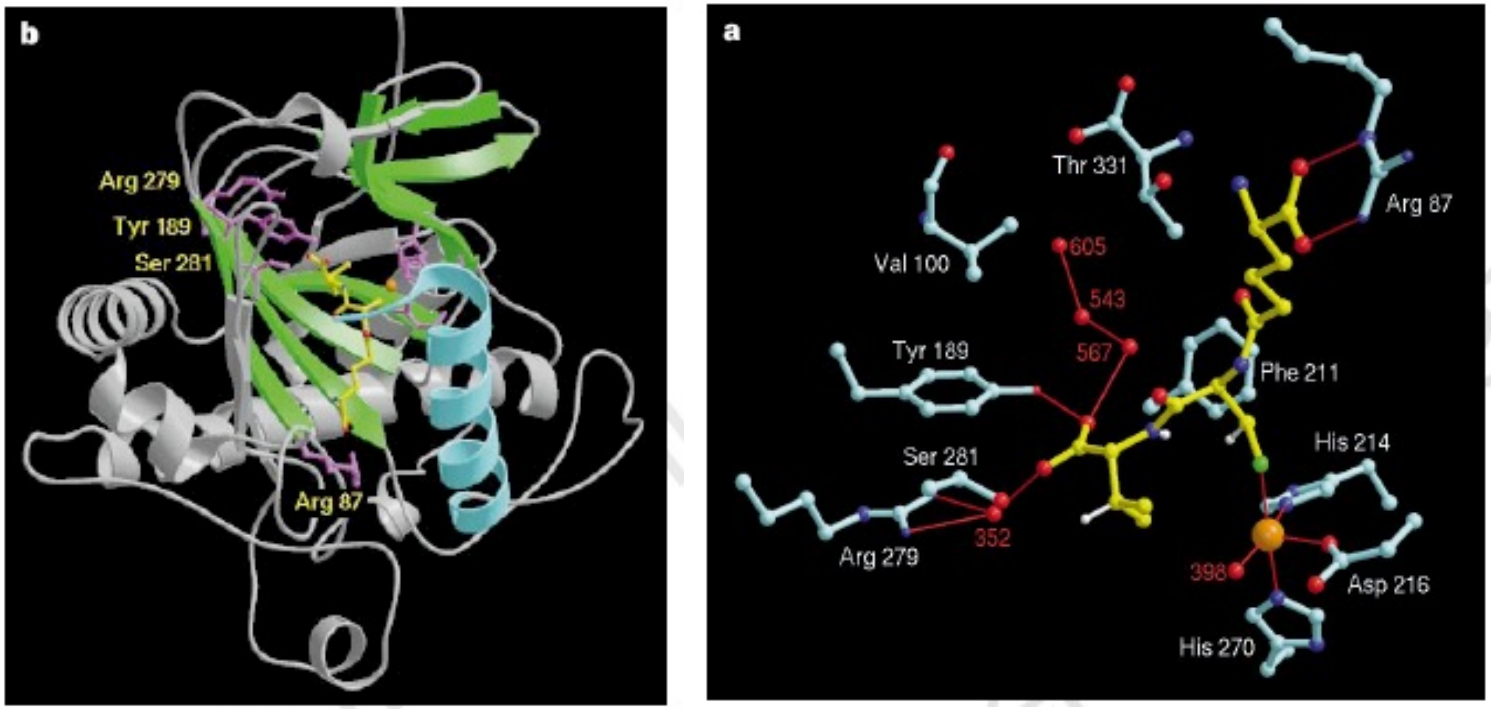
The C* domain may be involved in recruiting and/or positioning the substrate for the atypical reaction

Structure of isopenicillin N synthase (IPNS) and deacetoxycephalosporin synthase (DAOCS)



IPNS and DAOCS catalyze the reactions of closure and expansion of the β-lactam nucleus rings

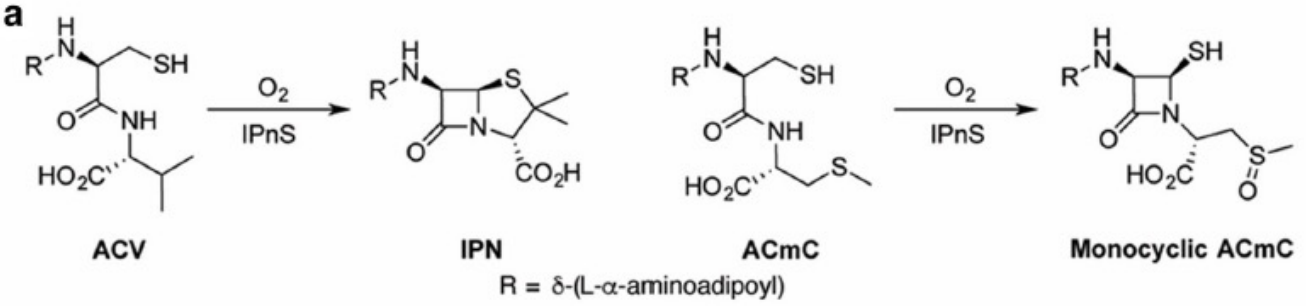
Structure of *A. nidulans* IPNS Active site of the enzyme



IPNS is an enzyme that uses iron and oxygen to catalyze synthesis of the β-lactam ring. The iron atom in the active site is coordinated by two histidine and an aspartate residue.

Reaction mechanism proposed for IPNS

Crystal structures of IPNS in the presence of substrate analogues that do not allow reaction completion.



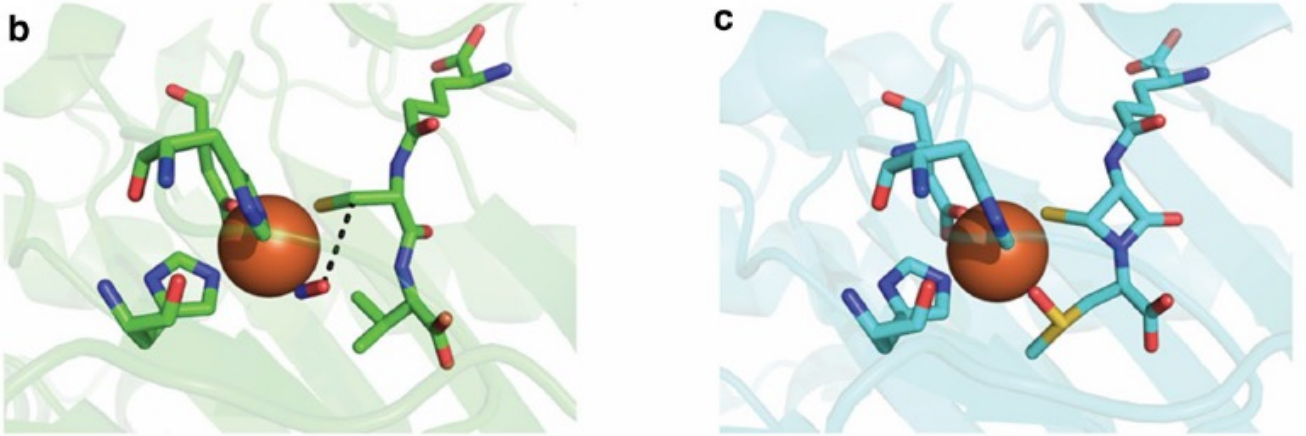
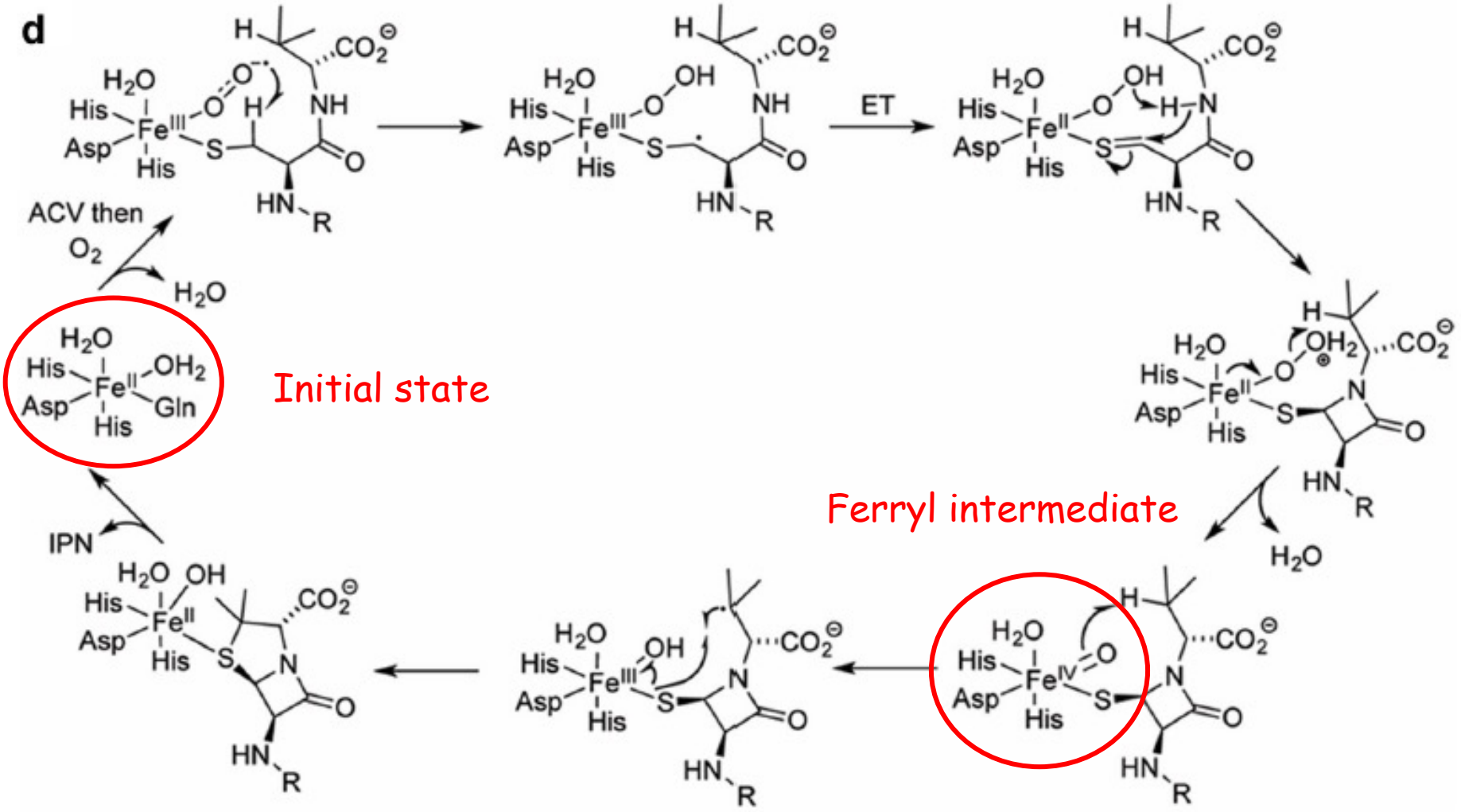
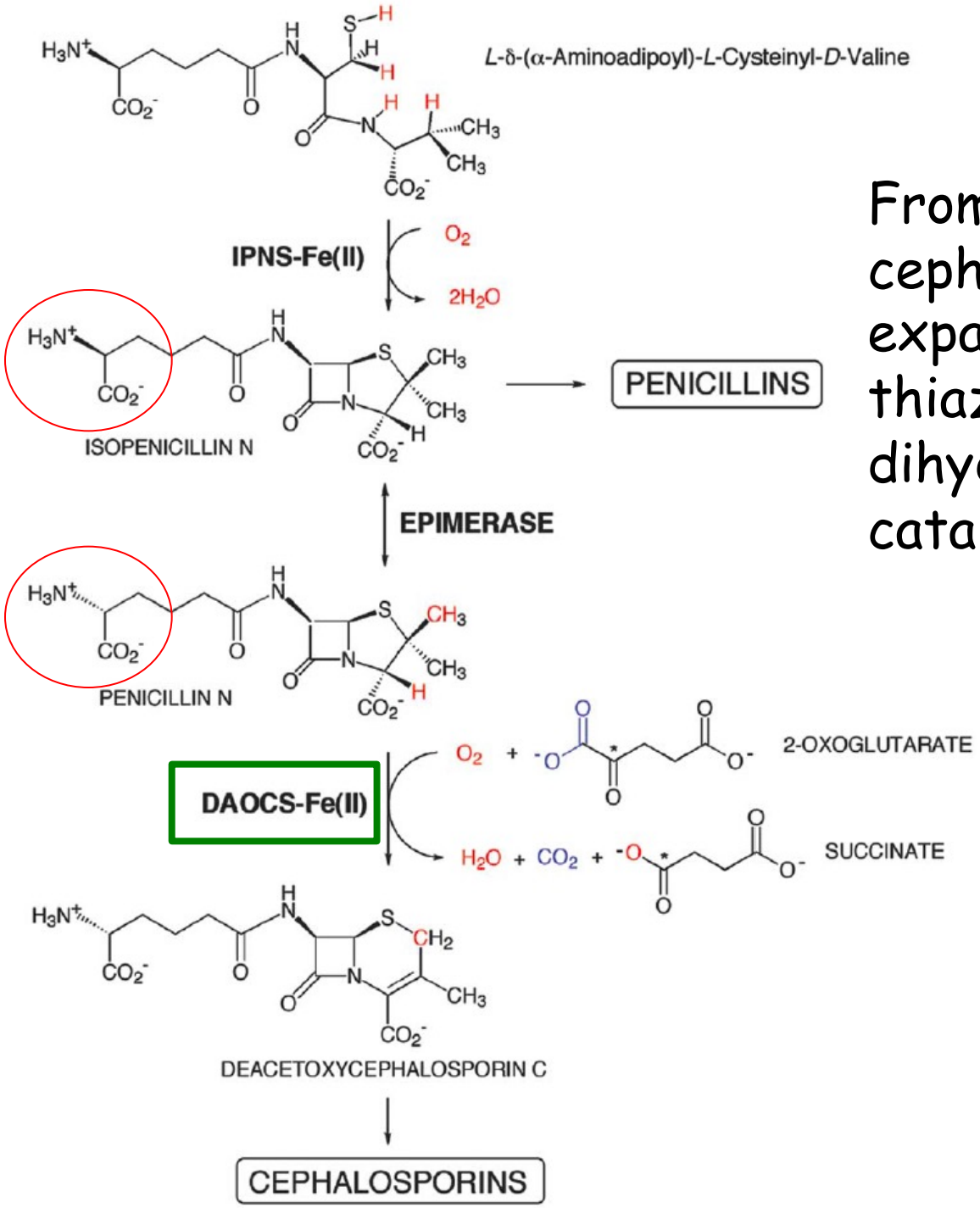


Fig. 2 Overview of the chemistry effected by IPNS. **a** The native substrate of IPNS catalysis (*left*) and a substrate analog that does not undergo a second cyclization (*right*). **b** The crystal structure of the ternary complex of Fe(II)-IPNS-ACV-NO (PDB ID: 1BLZ). The distance between the oxygen atom of NO to the β carbon of cysteine in ACV is 3.3 Å (*black dashes*). **c** The structure of the product of the in crystallo reaction of Fe(II)-IPNS with ACmC reveals formation of the thiazolidine ring and sulfoxidation of the methylcysteine moiety (PDB ID: 1QJF). **d** A proposed mechanism for catalysis by IPNS. Instead of a formal ET in the second step after substrate binding, the two structures could also be considered resonance forms. For details, see the text

Reaction mechanism proposed for IPNS

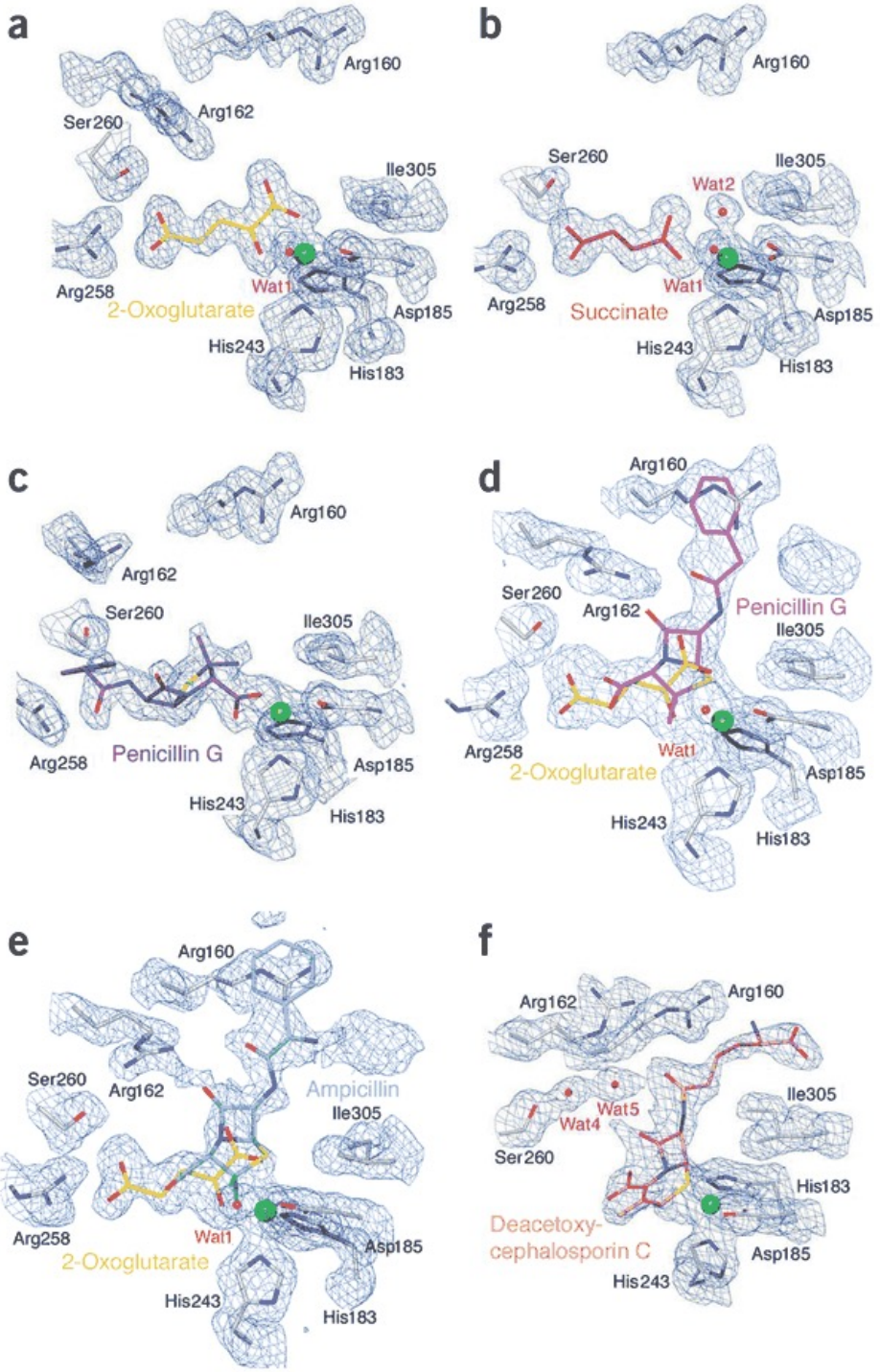




From penicillin to cephalosporin: expansion of the thiazolydine ring to dihydrothiazine ring catalyzed by DAOCS

Reaction mechanism of DAOCS, an iron- and α-chetoglutarate-dependent enzyme.

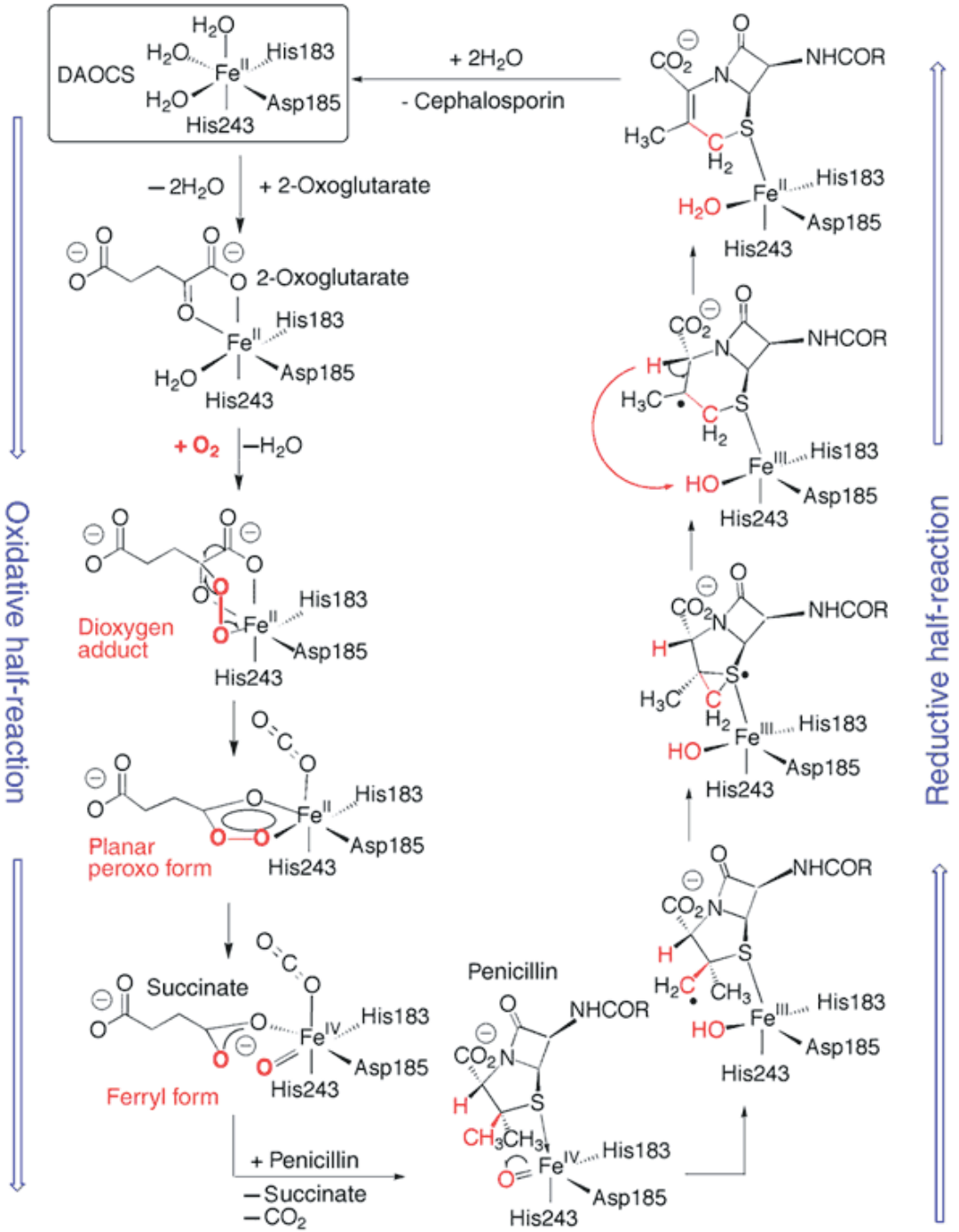
The binding site for penicillin partially overlaps the binding site for α-chetoglutarate ➔ sequential reaction



The active site region of DAOCS in complex with substrates and products.
(a) The DAOCS−Fe(II)−2-oxoglutarate complex at 1.5-Å resolution. (b) The DAOCS−Fe(II)−succinate complex at 1.5-Å resolution. (c) The DAOCS−Fe(II)−penicillin G complex at 1.6-Å resolution. (d) The DAOCS−Fe(II)−2-oxoglutarate−penicillin G complex at 1.7 Å resolution. (e) The DAOCS−Fe(II)−2-oxoglutarate−ampicillin complex at 1.5-Å resolution. (f) The DAOCS−Fe(II)−DAOC complex at 1.7-Å resolution. The density next to the penicillin side chain in d,e corresponds to a minor alternative conformation of the side chain. Dioxygen is expected to bind at the position of Wat1 in a. The oxygen of the ferryl iron would be formed at this site. The carbon atoms in 2-oxoglutarate are yellow, in succinate orange, in penicillin G magenta, in ampicillin cyan and in DAOC gold.

Reaction mechanism of DAOCS

- Binding of α-chetoglutarate activates Fe allowing binding of O_2
- Oxidative decarboxylation of the co-substrate gives rise to an oxidizing intermediate stabilized by succinate
- When penicillin expels succinate it triggers oxidative attack
- Formation of a radical and transfer of 2 electrons to oxygen produce cephalosporin and H_2O



Strains for the industrial production of penicillin

Table 1 Penicillin titre various organisms

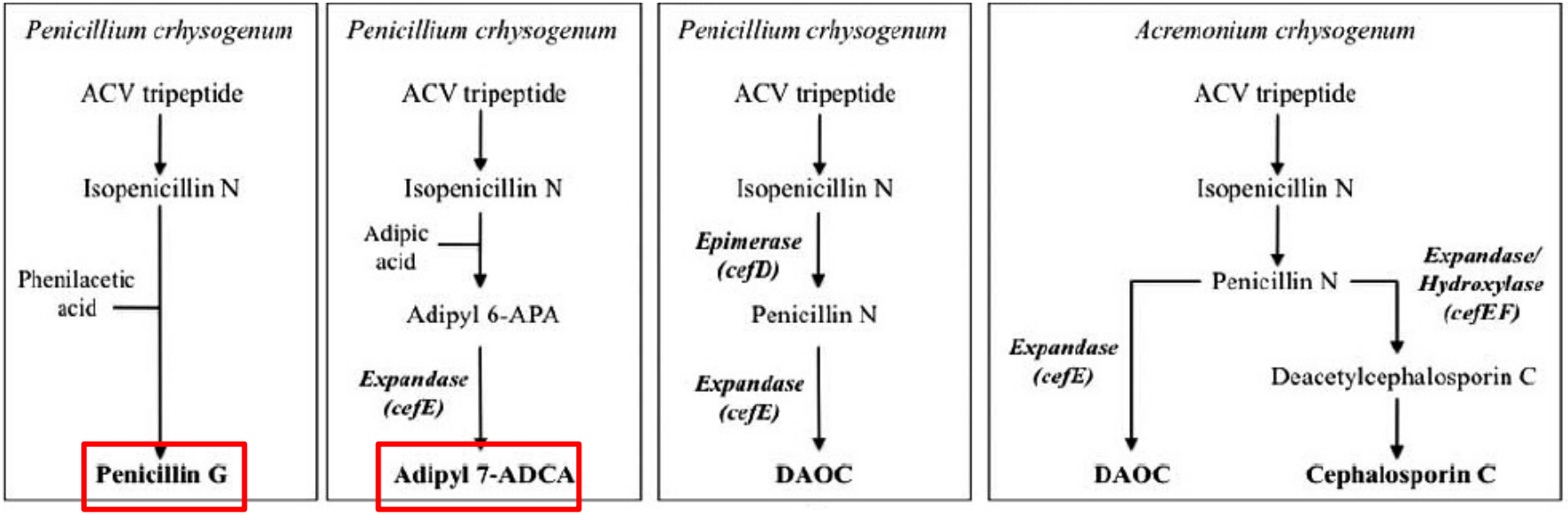
Species	Strain	Origin	Penicillin titre (g/l)	Reference
Aspergillus nidulans	7/142	Isolate	0.003	Simpson and Caten 1979
	A6-9	6 rounds CSI	0.012	Simpson and Caten 1979
Aspergillus oryzae	NS4DLDP	Isolate	0.00005	Marui et al. 2010
	OE-A	Transformant with 3 genes	0.00098	Marui et al. 2010
Saccharomyces cerevisiae	HpPen4	Transformant with 5 genes	0.001	Gidijala et al. 2009
Penicillium nalgiovense	Various	Food isolates	0.01–0.065	Laich et al. 2002
Penicillium griseofulvum	Various	Sausage isolates	0.04	Laich et al. 1999
Penicillium notatum	NRRL1249B1	Fleming's isolate	0.0012	Jami et al. 2010a
Penicillium chrysogenum	NRRL1951	Isolate cantaloupe	0.0155–0.150	Smidák et al. 2010b
				Jami et al. 2010a
	Wisconsin54-1255	Derivative of NRRL1951 (16 generations)	0.02	Kiel et al. 2005
			0.550–0.8	Jami et al. 2010a
	DS08425	Derivative of Wisconsin54-1255 (9 generations)	0.98	Kiel et al. 2005
	DS04825-PEX11	Pex11 overexpression in DS04825	2.03	Kiel et al. 2005
	AS-P-78	Derivative of Wisconsin lineage	4.8	Jami et al. 2010a
	BW1952	Derivative of Wisconsin lineage	~10	Newbert et al. 1997
	NMU2/40	Derivative of Wisconsin lineage	6	Smidák et al. 2010b
	Best industrial strains	Derivative of Wisconsin lineage	30–55	Lein 1986
				Rowlands 1991

Table 2 Changes in penicillin manufacturing technology

Fermentation	1950	2000
Carbon source	Lactose	Glucose/sucrose
Operational mode	Batch	Fed-batch
Medium sterilization	Batch	Continuous
Air filtration	Depth filters	Membrane filters
Feeds	None	Many
Morphology	Filamentous	Pelleted
Cycle time	120 h	120–200 h
Tank volume (1,000 gallons)	10–20	20–60
Assay	Bio-assay	HPLC
Control	Temperature only	Computerized
Titer (g/l)	0.5–1.0	>40
Recovery and purification		
Mycelium removal	Filtration	Whole broth
Operational mode	Batch	Semi-continuous
Extraction stages	Many	Single to few
Precursor recovery and re-use	Discarded	Recovered and re-used
Efficiency (%)	70–80	>90
Environmental issues	Few	Many
Bulk cost	~US$275–350/kg	~US$15–20/kg

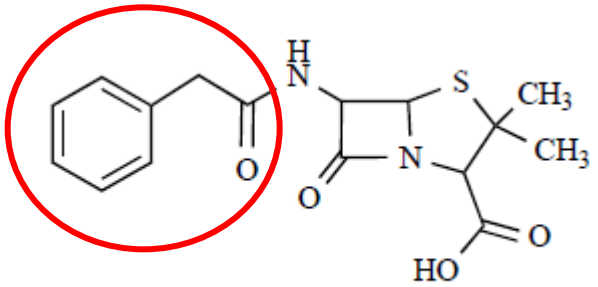
Production of penicillin and cephalosporin

- Strains of Penicillium chrysogenum: yields up to about 70 gr penicillin G/lt of culture
- Aerobic fermentation
  - Carbon sources: glucose and corn steep liquor
  - Nitrogen source: ammonia
  - Addition of phenylacetic acid in stationary phase



Semi-synthetic penicillins

- Semi-synthetic penicillins are obtained from natural penicillin G
- The side-chain is removed from penicillin G producing 6-aminopenicillanic acid (6-APA)
- 6-APA is acylated with a new side-chain
- β-lactam acylases are enzymes able to catalyze both reactions



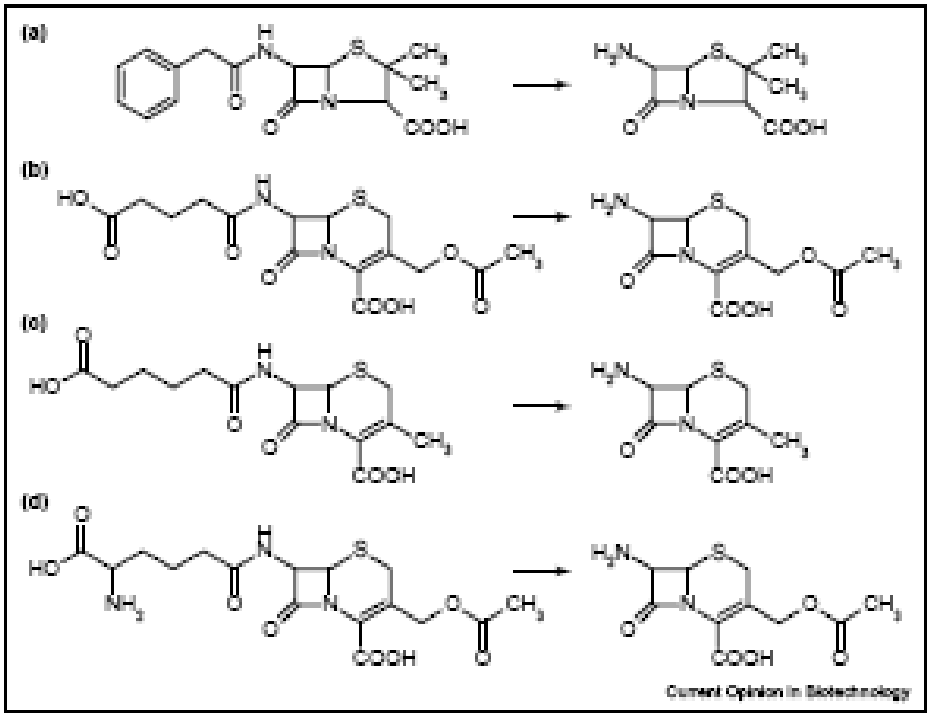
Penicillin G

Hydrolysis reactions catalyzed by β-lactam acylases: removal of the acylic side-chain

Penicillin acylase hydrolyzes penicillin G to 6-aminopenicillanic acid (a).

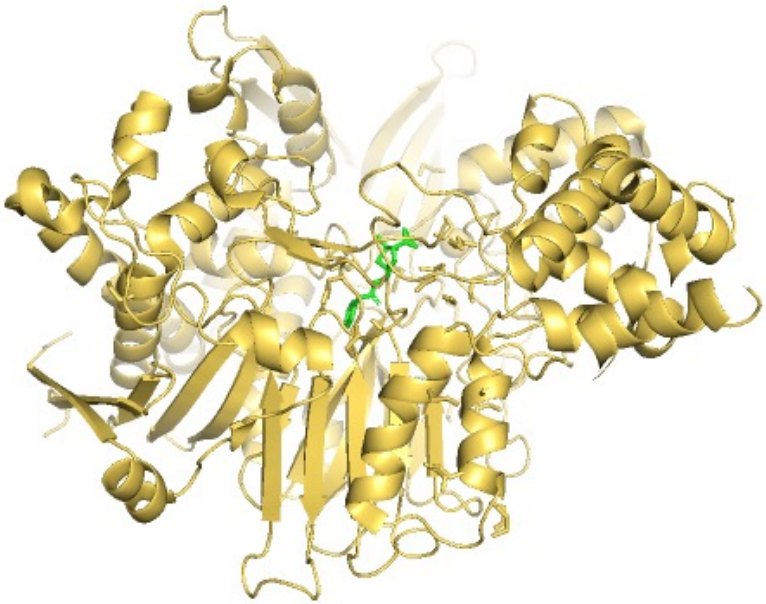
Cephalosporin acylase hydrolyzes glutaryl-7-ACA to 7-ACA (b).

Cephalosporin acylase mutants hydrolyze adipyl-7-ADCA to 7-ADCA (c) and cephalosporin C to 7-ACA (d).

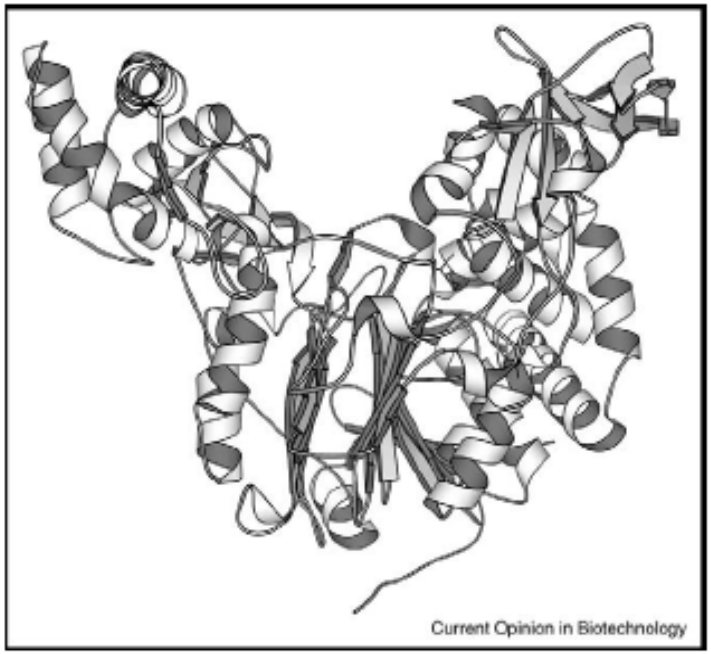


6-APA and 7-ACA are the precursors of semi-synthetic penicillins and cephalosporins

Structure of penicillin acylase and cephalosporin acylase



Structure of *Escherichia coli* penicillin acylase

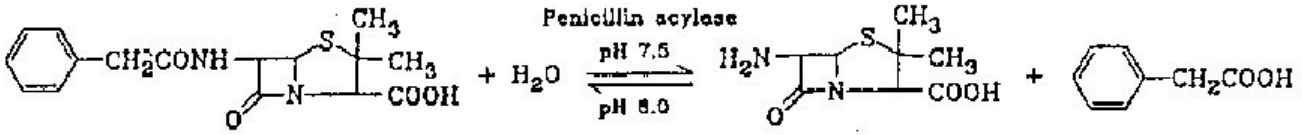


The crystal structure of *Pseudomonas diminuta* cephalosporin acylase [11]. (Figure drawn with Molscript [27].)

Producing organisms: bacteria and fungi (*Escherichia coli, Bacillus megaterium, Kluyvera citrophila, Pseudomonas melanogenum, Penicillium chrysogenum*).

Penicillin acylase

- Penicillin acylase catalyzes hydrolysis of penicillin with formation of 6-aminopenicillanic acid (6-APA), precursor of semi-synthetic penicillins.
- The reaction is reversible and the direction depends on pH.
- Substrates: penicillins, some cephalosporins, amides and esters.
- Used for production of semi-synthetic penicillins, both for production of 6-APA and for the synthesis reaction.



Penicillin acylase reaction.

Penicillin acylase

- *E. coli* penicillin acylase is formed by two subunits α (24 kDa) and β (62 kDa) derived from a 95 kDa precursor, which is proteolytically processed by removal of
  - a leader peptide of 26 aa necessary for periplasm targeting
  - an internal 54 aa peptide that activates the enzyme.
- Substrate specificity (penicillin side-chain) is found in the α subunit, while in the β subunit catalytically active serine B1 is found.

Reaction mechanism of penicillin acylase

- Serina B1 is activated as nucleophile by its free amino group.
- The mechanism of catalysis involves a covalent acyl-enzyme intermediate.
- The acyl-enzyme intermediate is deacylated by a water molecule.

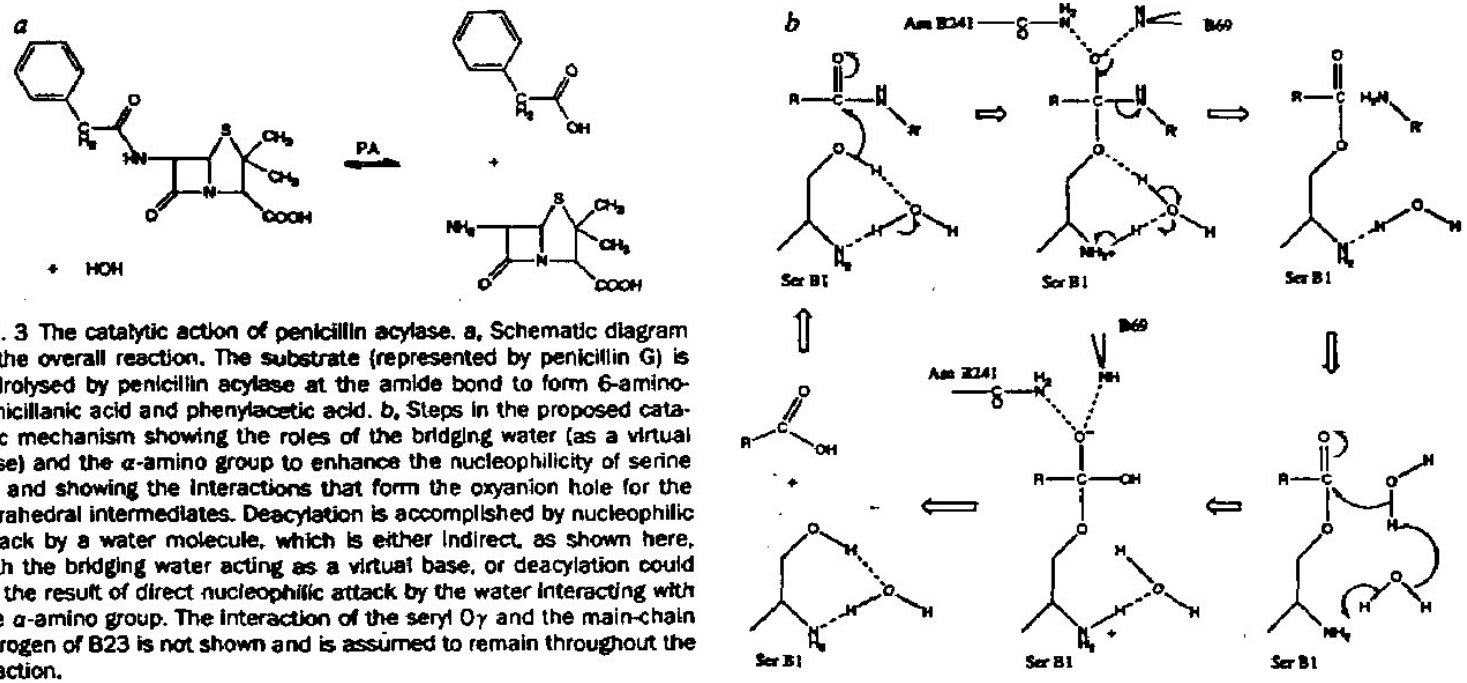
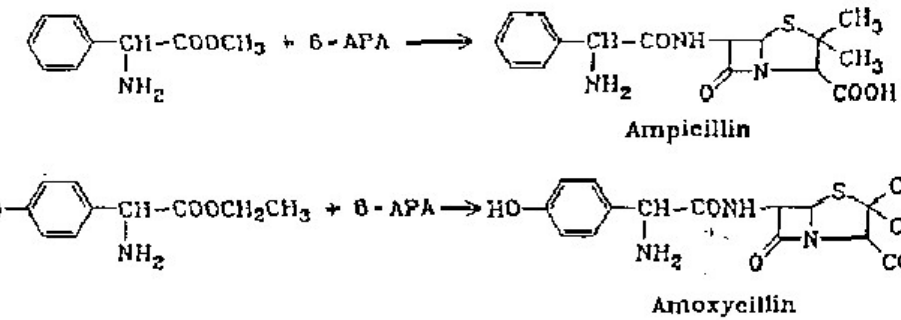


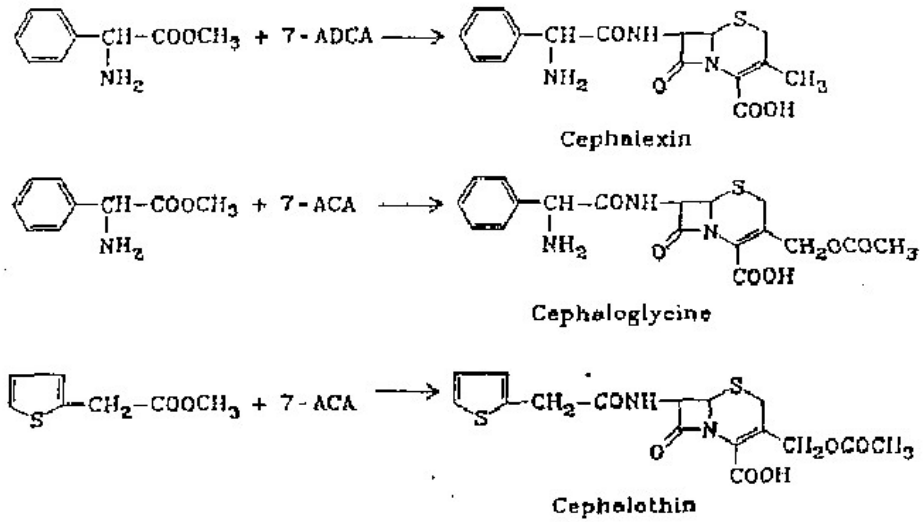
FIG. 3 The catalytic action of penicillin acylase. a, Schematic diagram of the overall reaction. The substrate (represented by penicillin G) is hydrolysed by penicillin acylase at the amide bond to form 6-amino-penicillanic acid and phenylacetic acid. b, Steps in the proposed catalytic mechanism showing the roles of the bridging water (as a virtual base) and the α-amino group to enhance the nucleophilicity of serine B1 and showing the interactions that form the oxyanion hole for the tetrahedral intermediates. Deacylation is accomplished by nucleophilic attack by a water molecule, which is either indirect, as shown here, with the bridging water acting as a virtual base, or deacylation could be the result of direct nucleophilic attack by the water interacting with the α-amino group. The interaction of the seryl Oγ and the main-chain nitrogen of B23 is not shown and is assumed to remain throughout the reaction.

Production of semi-synthetic β-lactams by Penicillin acylase

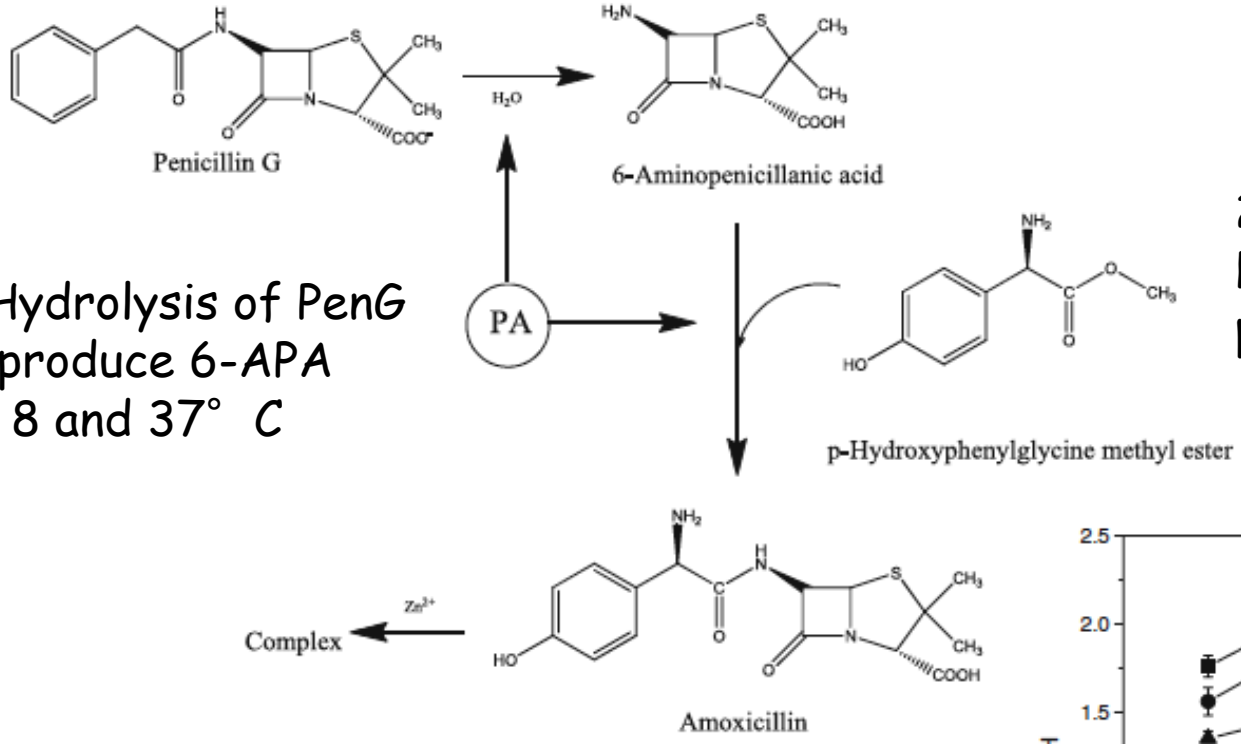
Synthesis of semi-synthetic penicillins from **esters** of the side-chain R (acyl donors) and 6-APA.



Synthesis of semi-synthetic cephalosporins from **esters** of the side-chain R (acyl donors) and 7-ACA.



Synthesis of amoxycillin



1. Hydrolysis of PenG to produce 6-APA
pH 8 and 37° C

2. Addition of HPGME and $ZnSO_4$
pH 6 and 25° C

Fig. 1 Scheme of one-pot enzymatic synthesis of amoxicillin in the presence of zinc ions

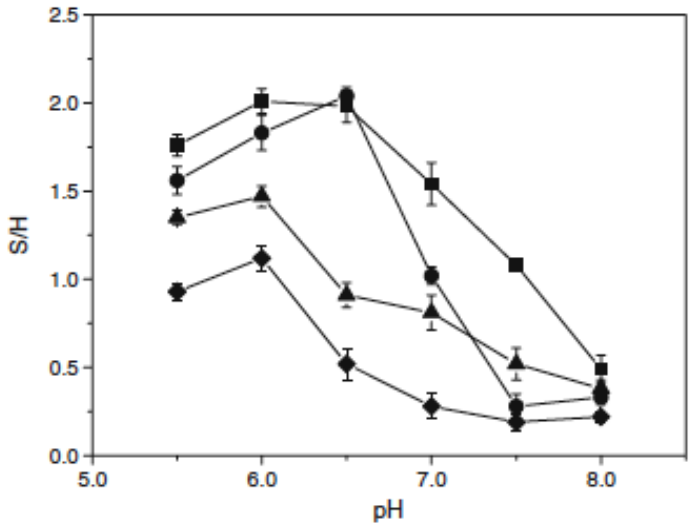


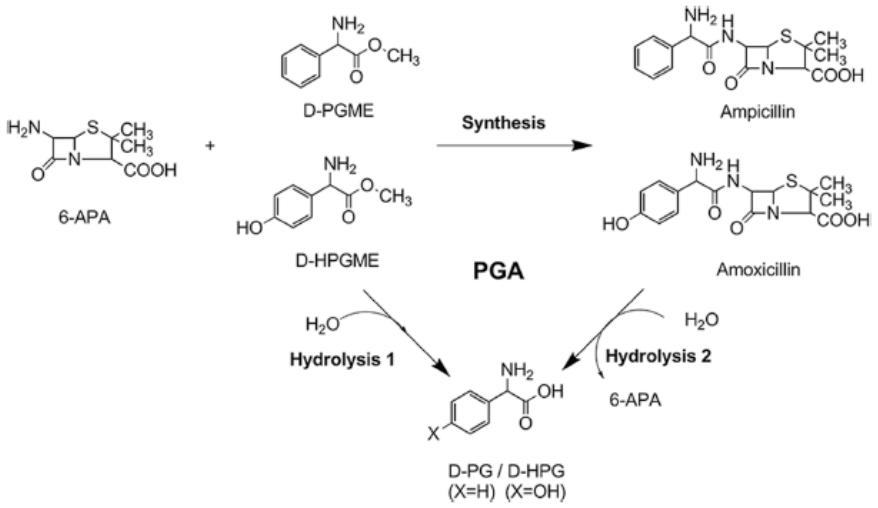
Fig. 4 Effect of pH on the S/H of the immobilized penicillin acylase. *Square*: AfPA; *circle*: KcPA; *upright triangle*: PCA; *diamond*: IPA

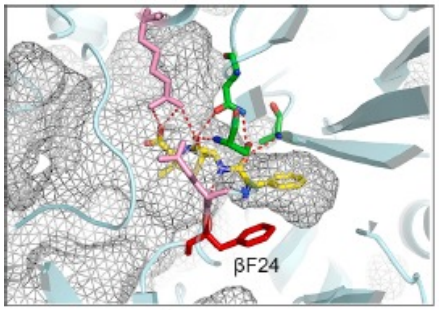
In the presence of zinc amoxycillin precipitates, favouring the synthesis reaction

Engineering of penicillin acylase to improve synthesis

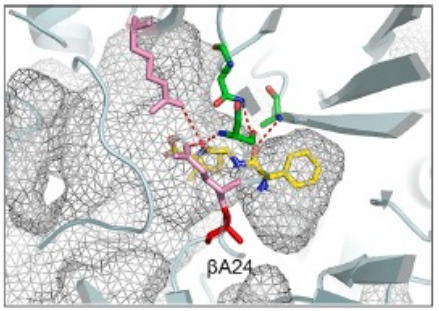
Hydrolysis of the acyl donor or of the product decrease synthesis yields

Increasing the ratio $V_{synthesis}/V_{hydrolysis}$: β F24 and α F146
the enzyme must have a higher affinity for the acyl donor compared to the antibiotic (product)

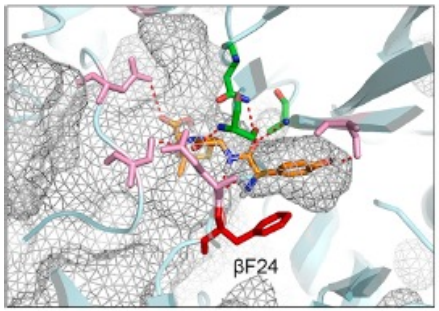




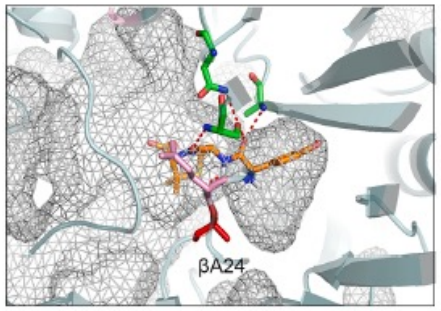
A (Ampicillin)



B (Ampicillin)



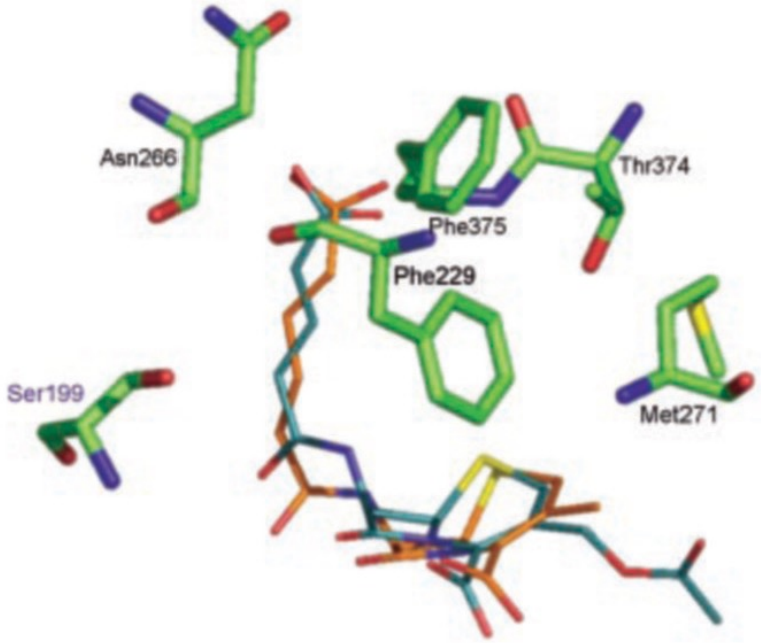
C (Amoxicillin)

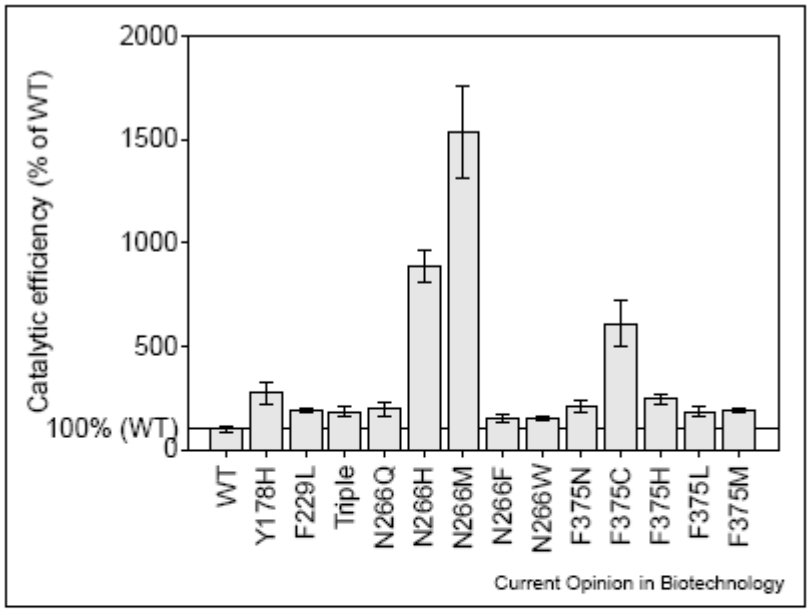


D (Amoxicillin)

Engineering of cephalosporin acylase

Altering substrate specificity to improve hydrolysis of cephalosporin C and adipyl-7-ADCA for production of 7-ACA and 7-ADCA





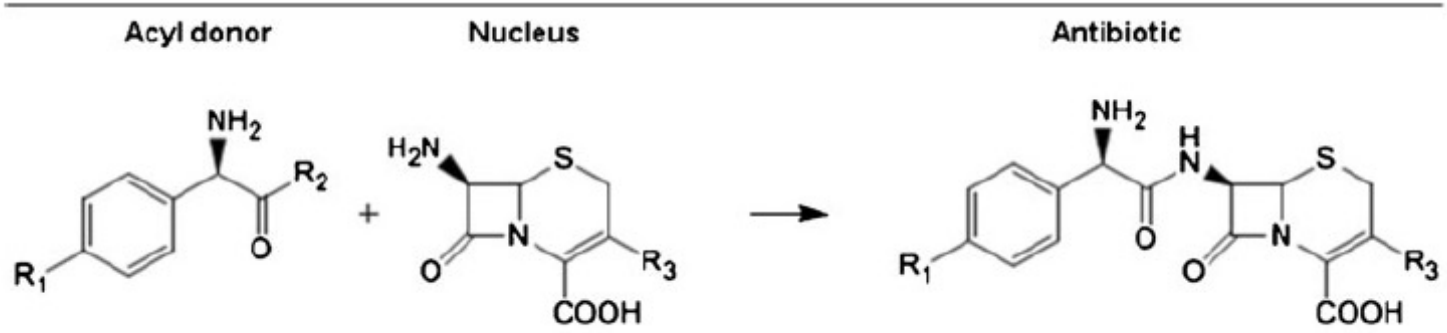
Mutagenesis alters the substrate specificity of a cephalosporin acylase. The preferred substrate of cephalosporin acylase is glutaryl-7-ACA and activity towards the novel β-lactam fermentation product adipyl-7-ADCA is much lower. Mutants that exhibit an increased catalytic efficiency towards adipyl-7-ADCA were obtained by directed evolution of a cephalosporin acylase. Shown here are the best mutants found by random mutagenesis [17,18••] and saturation mutagenesis of selected residues [20,21]. A greater than 15-fold increase in activity was observed for the single mutant N266M (in single-letter amino acid code). WT, wild type; triple, the multiple mutant M271V/Q291K/T374S.

Appl Microbiol Biotechnol (2012) 95:1491–1500
DOI 10.1007/s00253-011-3817-y

BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Efficient biocatalyst for large-scale synthesis of cephalosporins, obtained by combining immobilization and site-directed mutagenesis of penicillin acylase

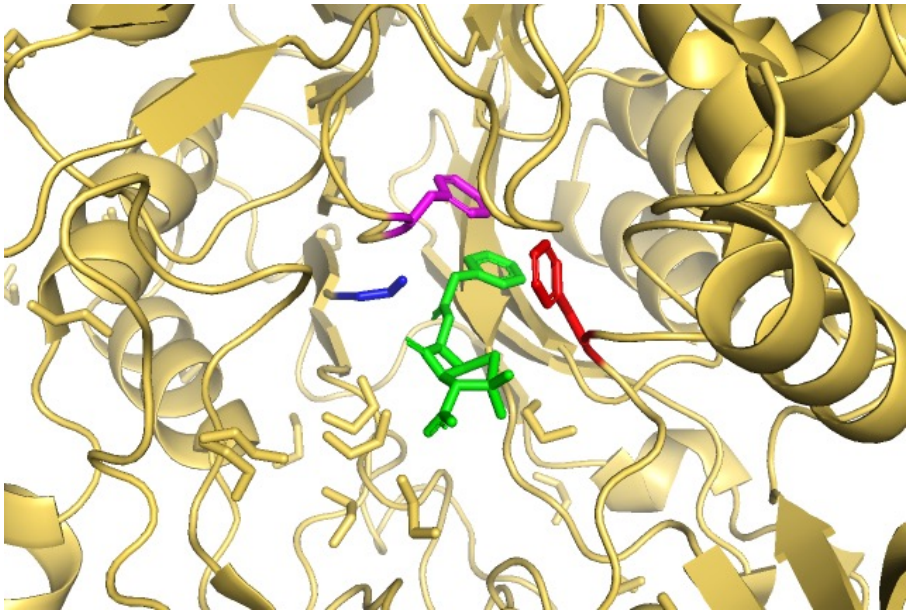
Davide A. Cecchini · Roberto Pavesi · Sara Sanna · Simona Daly · Roberto Xaiz · Massimo Pregnolato · Marco Terreni



Compound	R_1	R_2	Compound	R_3	
D-PGA or D-PGME	H	NH_2	7-ADCA	CH_3	Cephalexin
	H	OCH_3	7-ACCA	Cl	Cefaclor
D-HPGME	OH	OCH_3	7-APRA	$CH{=}CH{-}CH_2$	Cefprozil

Penicillin acylase engineering

- Identification of βF24 and αF146 involved in substrate recognition.
- Substitution βF24A increases V_S/V_H ratio (low amidase activity).
- Substitution αF146Y retains high activity towards the acyl donor.
- Covalent immobilization of the biocatalyst on Eupergit C.



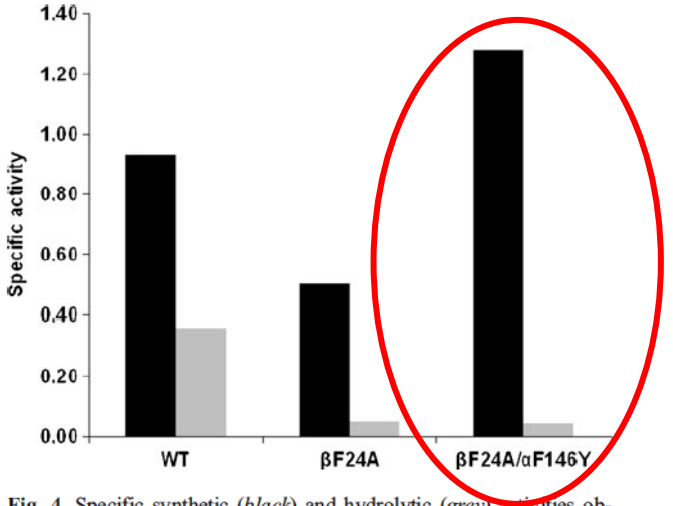


Fig. 4 Specific synthetic (*black*) and hydrolytic (*grey*) activities observed in the reactions performed using the immobilized WT and mutated acylases at 28 °C. Synthesis was performed with D-PGME. Activities are expressed as micromole per minute (μmol min^{-1}) of cephalexin synthesized or hydrolyzed per milligram of enzyme

Table 3 Loading capacity of glyoxyl Eupergit C250L toward WT-PA and immobilization of PA mutants at the optimal enzyme concentration

Enzyme	Specific activity (U_{PGK}/mg)	Offered enzyme (mg/g)	Yields (%)[a]	Activity (%)[b]	Derivative specific activity[c]
WT	38	5	42	80 (42%)	36
WT	38	10	38	140 (37%)	37
WT	38	15	30	160 (30%)	36
βF24A	11	10	38	18 (16%)	4.7
βF24A/αF146Y	0.7	10	40	2.3 (33%)	0.6

The results reported are the average of three experiments (SD<10%)

[a] Percentage of the total protein evaluated by Bradford assay

[b] Activity toward penicillin G potassium salt expressed per gram of support (U_{PGK}/g) and percentage of activity retained after immobilization

[c] Specific activity of the immobilized protein expressed as U_{PGK}/mg of protein

Table 4 Enzymatic synthesis of cephalexin, cefaclor and cefprozil catalyzed by the immobilized wild type and PA mutants with different activated acyl donors and at different temperatures

Enzyme	Derivative (g)	Acyl donor	Product	Temperature (°C)	vs[a] (μmol min^{-1})	Time[b] (min)	Conversion (%)
WT	1.5	D-PGA	Cephalexin	28	4.4	20	67
βF24A	1.5		Cephalexin	28	0.1	630	68
βF24A/αF146Y	1.5		Cephalexin	28	0.3	570	77
WT	1	D-PGME	Cephalexin	4	0.4	240	90
	1		Cephalexin	28	3.7	30	76
βF24A	1		Cephalexin	4	0.3	360	99
	1		Cephalexin	28	1.9	60	98
βF24A/αF146Y	1		Cephalexin	4	0.8	240	99
	1		Cephalexin	28	4.0	40	99
WT	1	D-PGME	Cefaclor	4	0.4	300	84
	1		Cefaclor	28	2.0	50	65
βF24A	1		Cefaclor	4	0.1	1,500	98
	1		Cefaclor	28	1.5	140	98
βF24A/αF146Y	1		Cefaclor	4	0.5	360	99
	1		Cefaclor	28	3.7	60	99
WT	1	D-HPGME	Cefprozil	28	0.2	160	59
βF24A	1		Cefprozil	28	0.5	100	98
βF24A/αF146Y	1		Cefprozil	28	0.6	100	99

[a] Reaction rate (vs) was measured monitoring the formation of the acylation product (μmol min^{-1}) by HPLC analysis

[b] Time employed to reach the maximal conversion

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Efficient cascade synthesis of ampicillin from penicillin G potassium salt using wild and mutant penicillin G acylase from *Alcaligenes faecalis*

Senwen Deng[a,c,1], Xiaoqiang Ma[c,1], Erzheng Su[b,*], Dongzhi Wei[c,**]

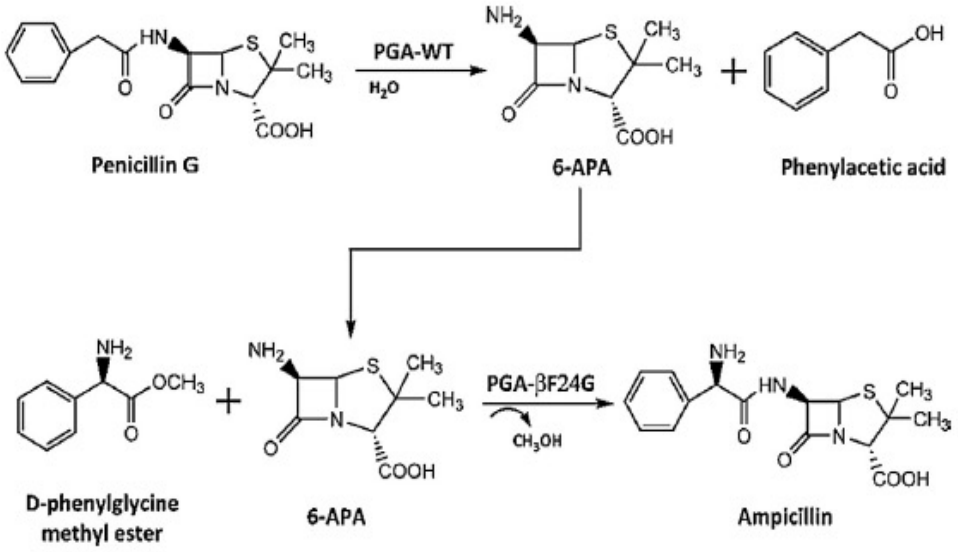


Fig. 1. Scheme of two-enzyme two-step cascade synthesis of ampicillin.

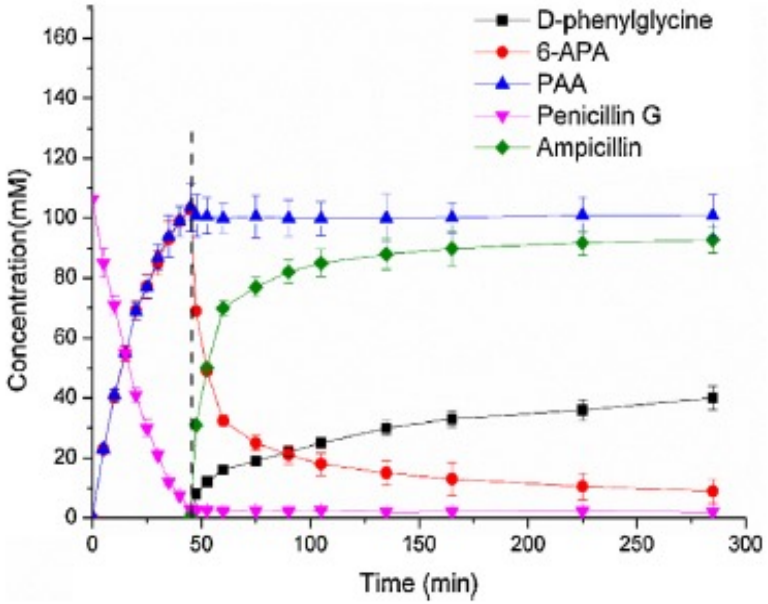


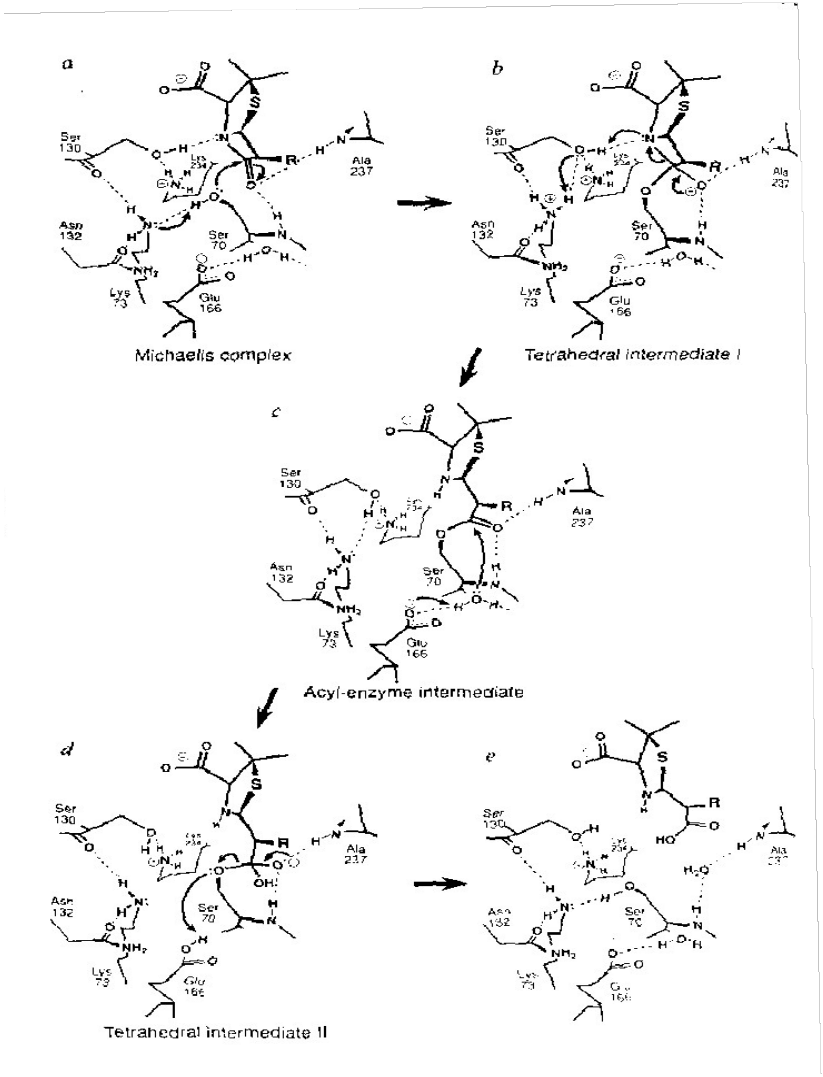
Fig. 6. Time course of two-enzyme two-step cascade synthesis of ampicillin. *Dotted line* is the demarcation line of the first step and the second step. Conditions of the first step: pH 8.0, 28 °C, 4% (w/v) PGK, 0.03 g/mL immobilized wild Af PGA; Conditions of the second step: initial pH 6.3, 28 °C, 150 mM D-PGME, 0.19 g/mL immobilized βF24 G mutant Af PGA.

Resistance to penicillin: Mechanism of class A β-lactamase

β-lactamases inactivate penicillin by catalyzing hydrolysis of the β-lactam ring.

Class A β-lactamase are serine-hydrolases.

Ser70 is the nucleophile that attacks the carbon atom of the β-lactam ring. Lys73 activates Ser70 moving the proton to Ser130, that transfers it to the nitrogen atom of the ring and opens it. An acyl-enzyme intermediate is form, that is deacylated by a water molecule, assisted by Glu166.



Structure of transition-state analogues that act as class A β-lactamase inhibitors

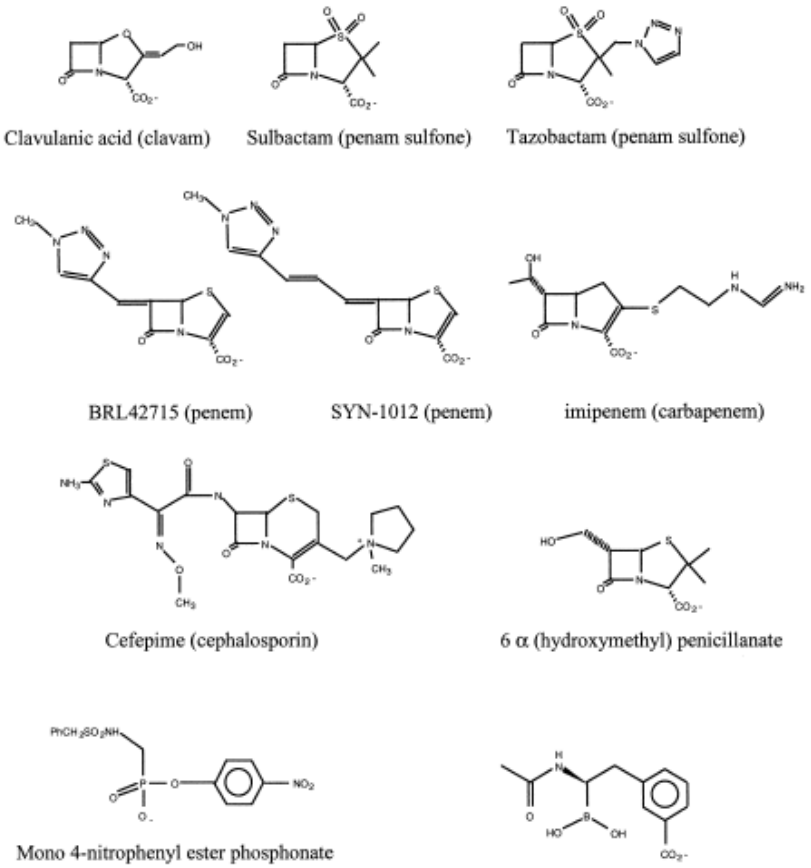


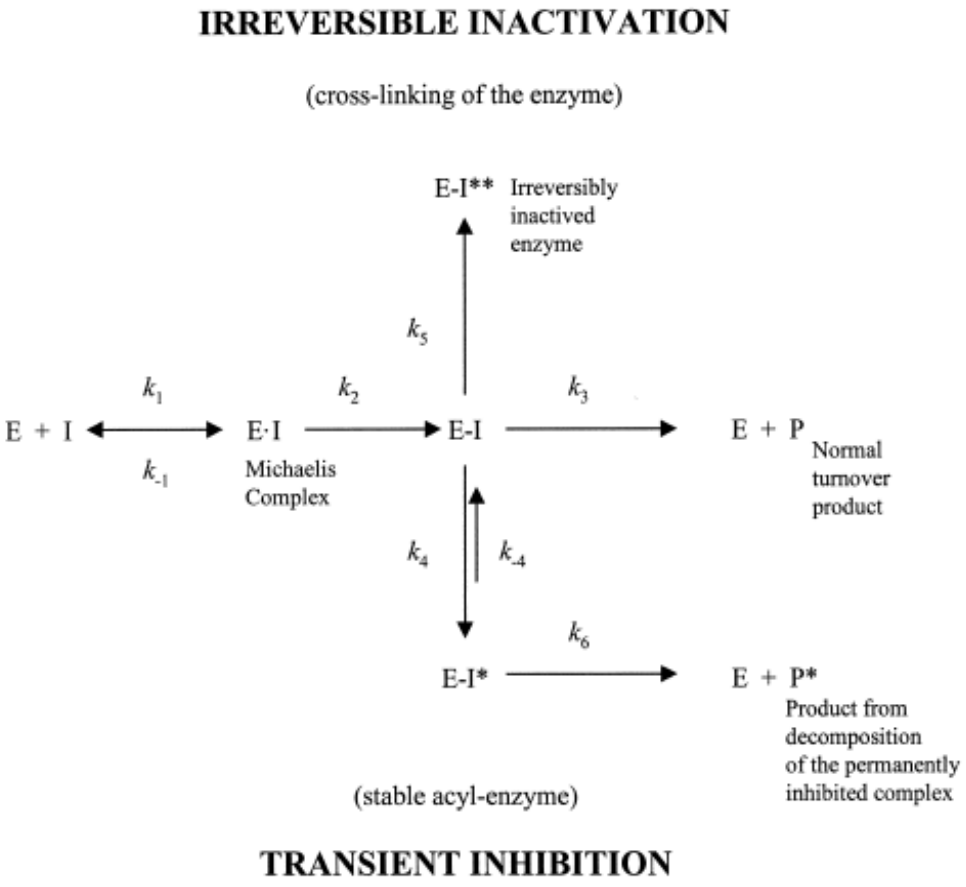
Fig. 1. Structure of β-lactams and transition state analogs with inhibitory activity against serine β-lactamases.

Scheme of the inactivation of β-lactamase

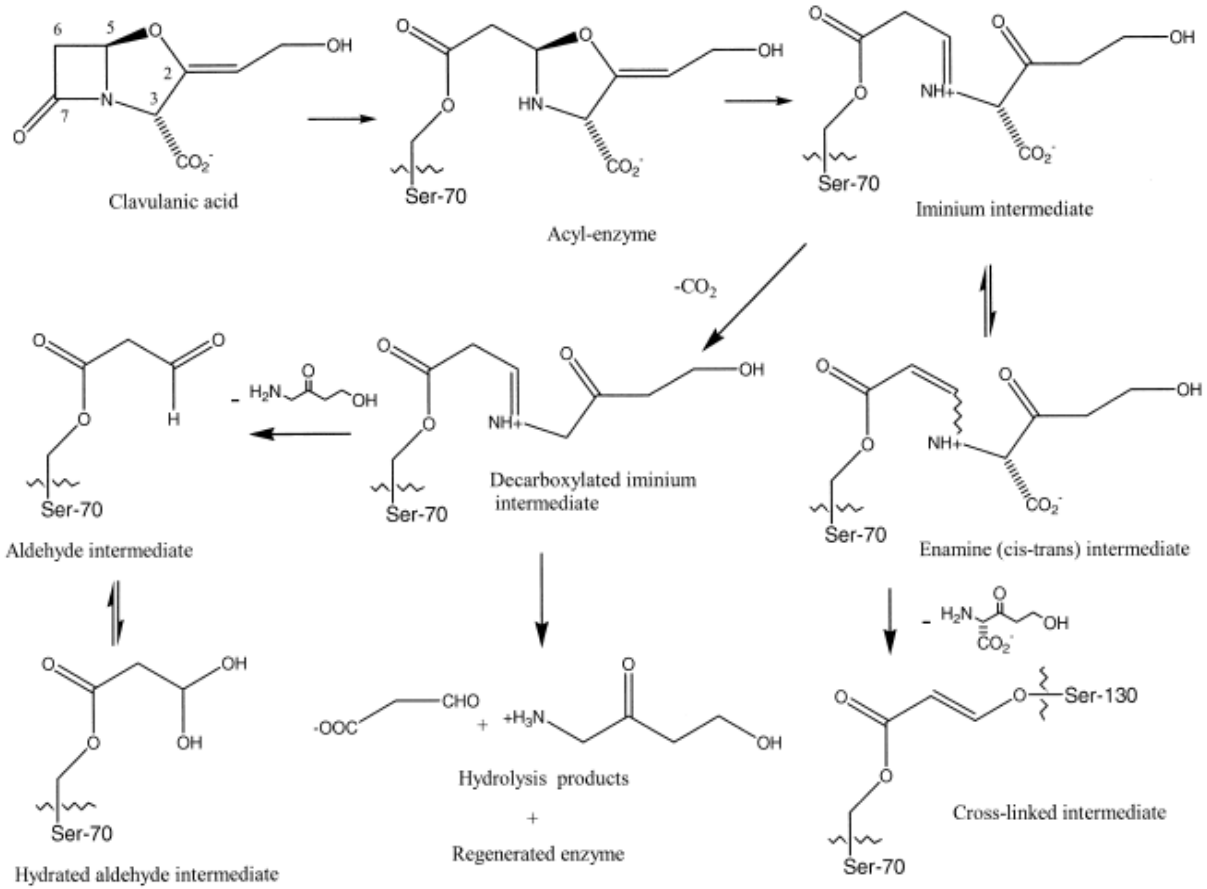
The acyl-enzyme intermediate can partition through two routes that lead to transient or irreversible inhibition.

Hydrolysis of the modified intermediate E-I* is very slow so it is possible to consider the enzyme as permanently inhibited.

Cross-linking leads to irreversible inactivation of the enzyme.



Mechanism of inactivation of class A β-lactamase by clavulanic acid, a suicide inhibitor.



Sultamicillin: a drug that potentiates the antibiotic (amoxycillin) with the β-lactamase inhibitor (sulbactam)

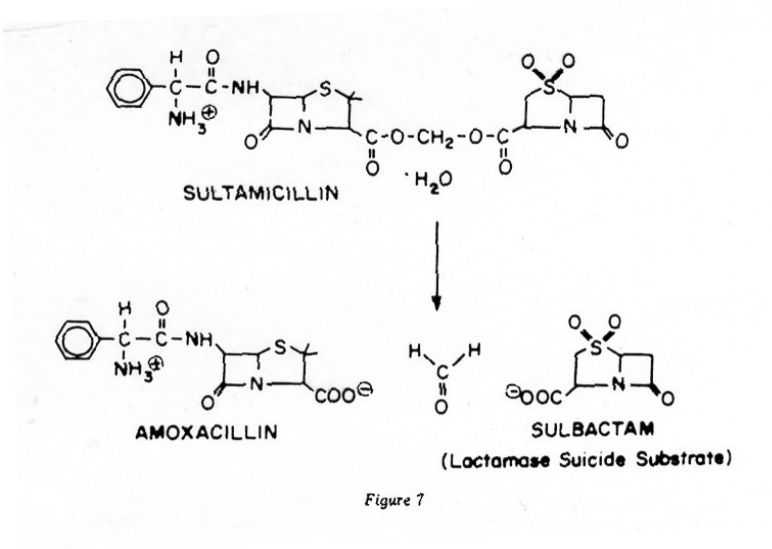
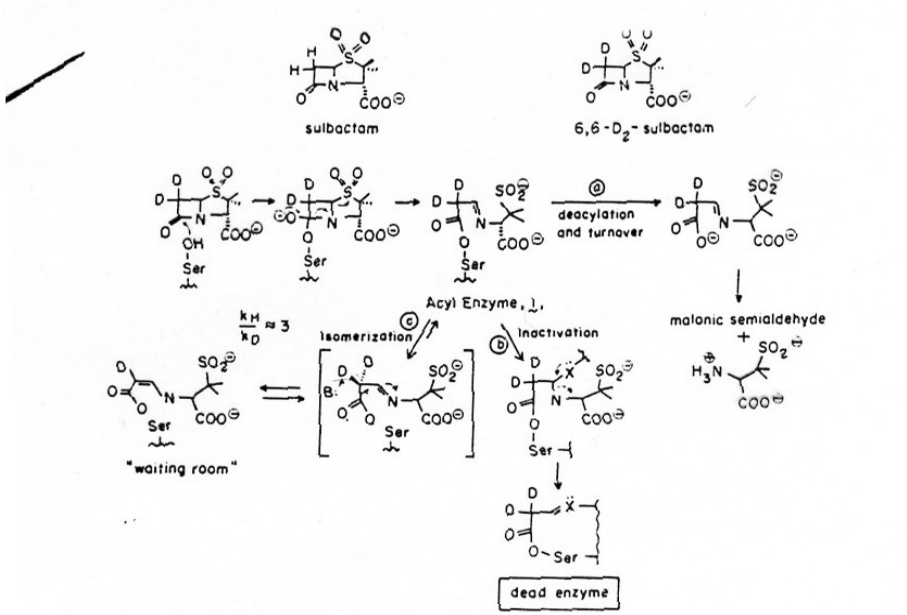


Figure 7

Mechanism of sulbactam



New inhibitors of class A β-lactamase

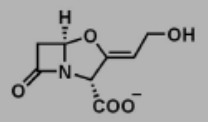
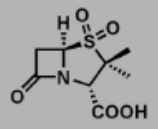
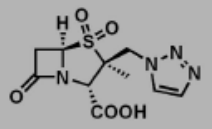
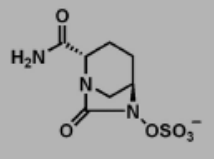
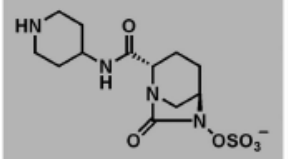
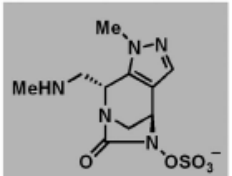
Generations:	1: Clavulanates	2: Penicillin Sulfones		3: Diazabicyclooctanes (DBOs)		
β-Lactamase Inhibitors						
	Clavulanate	Sulbactam	Tazobactam	Avibactam	MK-7655	NXL105
Development Status	Approved (Augmentin) GlaxoSmithKline	Approved (Cefobid) Pfizer	Approved (Zosyn) Pfizer	Phase II/III AstraZeneca/Forest	Phase I/II Merck	Preclinical AstraZeneca

Figure 6 Next generation 'non-β-lactam' β-lactamase inhibitors (diazabicyclooctanes; DBOs) in clinical development.

Ceftazidime-Avibactam: A Novel Cephalosporin/β-Lactamase Inhibitor Combination for the Treatment of Resistant Gram-negative Organisms

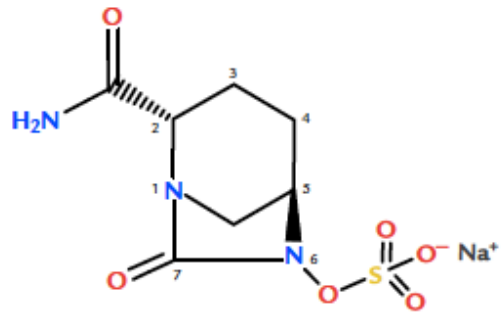


Figure 2. Chemical structure of avibactam sodium.

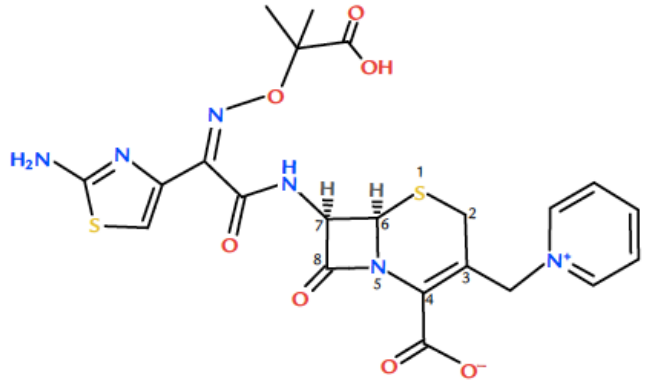


Figure 1. Chemical structure of ceftazidime.[11]