DESIGN AND SYNTHESIS OF NOVEL β-LACTAMS AND THEIR ANTIMICROBIAL ASSSAY

By:

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CERTIFICATE

This is to certify that the work titled **"SYNTHESIS OF NEW HETEROCYCLIC COMPOUNDS AS POSSIBLE ANTIMICROBIAL AGENTS"** submitted by **Bhavna Khattar (091754)** in partial fulfillment for the award of the degree of **Bachelor of Pharmacy** of Jaypee University of Information Technology, Waknaghat is a record of bona fide research work carried out by her under my supervision and guidance. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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A single flower cannot make a garland or a single star cannot make the beautiful shiny sky at the night. A project work can never be outcome of a single individual's talent or efforts.

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Bhavna Khattar

Bhavna Khattar

Date: 28th May, 2013

SUMMARY

Anti microbial drugs have caused a variety of drastic change not only in the treatment of infection but also in improvement in the human life. However, in reality, emerging and re-emerging infectious disease have lead scientists to discover and design new drugs with higher potency, less side effects and a broader spectrum of action with reduced toxicity.

With this objective, we designed the strategy to synthesize chemical compounds as possible antimicrobial agent using imines. Imines (compounds with double bond between carbon and nitrogen) were prepared using aldehyde and amine under laboratory conditions as specified. In selected imines an attempt to introduce a β-lactam ring were made to obtain β-lactam ring bearing compounds. The products were characterized on the basis of appearance (color, physical state), melting point and TLC monitoring (R_f value). Recrystallisation of products was performed to obtain pure compounds. Microbial studies was performed (antibiotic susceptibility testing by broth microdilution method) to study the antimicrobial activity of the prepared compounds.

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Chapter 1

Introduction

The treatment of bacterial infections is one of the few disease states that all clinicians are guaranteed to be challenged with at some time in their career. Because bacteria are constantly changing, the selection of appropriate antimicrobial therapy is crucial in providing efficacious treatment of infections. It is of the utmost importance that clinicians understand the medicinal chemistry of antimicrobial agents to choose the most effective therapy for their patients. Antimicrobial resistance trends are constantly changing and vary from institution to institution; therefore, a complete understanding of the structural relationship differences between antibiotics. even within the same class, is helpful when selecting the most appropriate therapy for an individual patient. In addition, side chains and subtle structural differences within antimicrobial classes can change the side effect profiles of these agents. The informed clinician can optimize treatment choices from patient to patient while avoiding severe adverse effects.

The development of newer and more effective antimicrobial agents is essential in the fight against infectious diseases. Understanding the medicinal chemistry of the older and newerantimicrobial agents helps the clinician to comprehend their differences in antimicrobial spectrum and side effect profile. Newer antibiotics are continuously being developed; however,

many of them are from antimicrobial classes already established with small changes on the functional groups and/or side chains, leading to dramatic differences in their antimicrobial spectra. As stated previously, bacteria are constantly changing, as are antimicrobial susceptibility patterns. To optimize patient care. the clinician needs to stay informed of the effects of the medicinal chemistry differences as newer drugs come to market and antimicrobialresistance trends change.

Resistance[1]

Resistance is the failure of microorganisms to be killed or inhibited by antimicrobial treatment. Resistance can either be intrinsic (be present before exposure to drug) or acquired (develop subsequent to exposure to a drug). Resistance of bacteria to the toxic effects of antimicrobialagents and to antibiotics develops fairly easily both in the laboratory and in the clinic and is an ever-increasing public health hazard. Challenging a culture in the laboratory with sublethal quantities of an antibiotic kills the most intrinsically sensitive percentage of the strains in thecolony. Those not killed or seriously

inhibited continue to grow and have access to the remainder of the nutrients. A mutation to lower sensitivity also enables individual bacteria to survive against the selecting pressure of the antimicrobial agent. If the culture is treated several times insuccession with sublethal doses in this manner, the concentration of antibiotic required to prevent growth becomes ever higher. When the origin of this form of resistance is explored, it is almost always found to be due to an alteration in the biochemistry of the colony so that the molecular target of the antibiotic has become less sensitive, or it can be due to decreased uptake of antibiotic into the cells. This is genomically preserved and passes to the next generation. The altered progeny may be weaker than the wild strain so that they die out if the antibiotic is not present to give them a competitive advantage. In some cases, additional compensatory mutations can occur that restore the vigor of the resistant organisms. Resistance of this type is usuallyexpressed toward other antibiotics with the same modeof action and thus is a familial characteristic; most tetracyclines,for example, show extensive cross-resistance withother agents in the tetracycline family. This is very enlighteningwith respect to discovery of the molecular mode ofaction but is not very relevant to the clinical situation.In the clinic, resistance more commonly takes place byresistance (R) factor mechanisms. In this case, enzymesare elaborated that attack the antibiotic and inactivate it.Mutations leading to resistance occur by many mechanisms.They can result from point mutations, insertions,deletions, inversions, duplications, and transpositionsof segments of genes or by acquisition of foreign DNAfrom plasmids, bacteriophages, and transposable geneticelements. The genetic material coding for this form ofresistance is often carried on extra chromosomal elementsconsisting of small circular DNA molecules knownas plasmids. A bacterial cell may have many plasmids ornone. The plasmid may carry DNA for several differentenzymes capable of destroying structurally dissimilarantibiotics. Such plasmid DNA may migrate within thecell from plasmid to plasmid or from plasmid to chromosomeby a process known as transposition. Such plasmidsmay migrate from cell to cell by conjugation (passagethrough a sexual pilus), transduction (carriage by a virusvector), or transformation (uptake of exogenous DNAfrom the environment). These mechanisms can convertan antibiotic-sensitive cell to an antibiotic-resistant cell.This can take place many times in a bacterium's alreadyshort generation time. The positive selecting pressure ofinadequate levels of an antibiotic favours explosive spreadof R-factor resistance. This provides a rationale for conservativebut aggressive application of appropriate antimicrobialchemotherapy.

Bacterial resistance is generallymediated through one of three mechanisms:

a) failure ofthe drug to penetrate in to or stay in the cell,

b) destructionof the drug by defensive enzymes, or

c) Alterations in thecellular target of the drug. It is rarely an ali-or-nothingeffect. In many cases, a resistant microorganism can still be controlled by achievable, although higher, doses thanare required to control sensitive populations.

Classification Scheme of Antibiotics[2]

Antibiotics

Bacterial Spectrum		Route of Administration		Type of Activity	
Broad	Narrow	Injectable	Oral	Bactericidal	Bacteriostatic

The first classification is according to the spectrum. The spectrum means the number of the organisms affected by the same drug. There are narrow and wide spectrum antibiotics. The wide spectrum antibiotics affect several types of bacteria and fungi and it is usually used where the specific type of the microorganism is unknown.

The second classification is according to the type of the action of antibiotics. It could be bactericidal or bacteriostatic. The bactericidal antibiotics kill the harmful microorganism while the baceriostatic ones tend to slow down their growth and give the body the chance to use its immune system against the microorganisms.

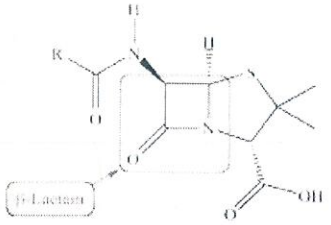
The third classification of antibiotics is according to the route of administration of the drug. The prevalent route of administration is the oral route but, there are other routes of administration that are more effective in certain cases like injection or topical applications.

Classification of Antibiotics According To Their Mechanism of Action And Chemical Structure[3]

Class (chemical structure)	Mechanism of action	Examples
B-lactam antibiotics ▪ Penicillins ▪ Cephalosporins ▪ Carbapenems	Inhibit bacterial cell wall synthesis	Penicillins ▪ Penicillin G ▪ Amoxicillin Cephalosporins ▪ Cefoxitin ▪ Cefotaxime Carbapenem ▪ Imipenem
Peptides	Inhibit bacterial cell wall synthesis	Bacitracin
Macrolides	Inhibit bacterial protein synthesis	▪ Erythromycin ▪ Azithromycin ▪ Clarithromycin
Tetracyclines	Inhibit bacterial protein synthesis	▪ Tetracycline ▪ Minocycline ▪ Doxycycline
Aminoglycosides	Inhibit bacterial protein synthesis	▪ Gentamicin ▪ Amikacin
Lincosamides	Inhibit bacterial protein synthesis	▪ Clindamycin ▪ Lincomycin
Fluoroquinolones	Inhibit bacterial DNA synthesis	▪ Norfloxacin ▪ Ciprofloxacin ▪ Ofloxacin
Imidazoles	Inhibit bacterial DNA synthesis	Metronidazole
Sulphonamides	Blocks bacterial cell metabolism by inhibiting enzymes	▪ Co-trimoxazole ▪ Trimethoprim

B-Lactam Antibiotics

β-Lactam antibiotic[4] are a broad class of antibiotics, consisting of all antibiotic agents that contains a β-lactam nucleus in its molecular structure.



a) β-Lactam antibiotics work by inhibiting cell wall synthesis of the bacterial organism and are the most widely used group of antibiotics.

b) β-Lactam antibiotics arebacteriocidal, and act by inhibiting the synthesis of the peptidoglycanlayer of bacterial cell walls. The peptidoglycan layer is important for cell wall structural integrity.

c) Bacteria often develop resistance to β-lactam antibiotics by synthesizing beta-lactamase, an enzyme that attacks the β-lactam ring. To overcome this resistance, β-lactam antibiotics are often given with β-lactamase inhibitorssuch as clavulanic acid, tazobactam, sulbactam.

d) However, with the development of antimicrobials, microorganisms have adapted and become resistant to previous antimicrobial agents. For this purpose, a new series of thiozolidin-4-ones were synthesized and evaluated for antimicrobial activity.

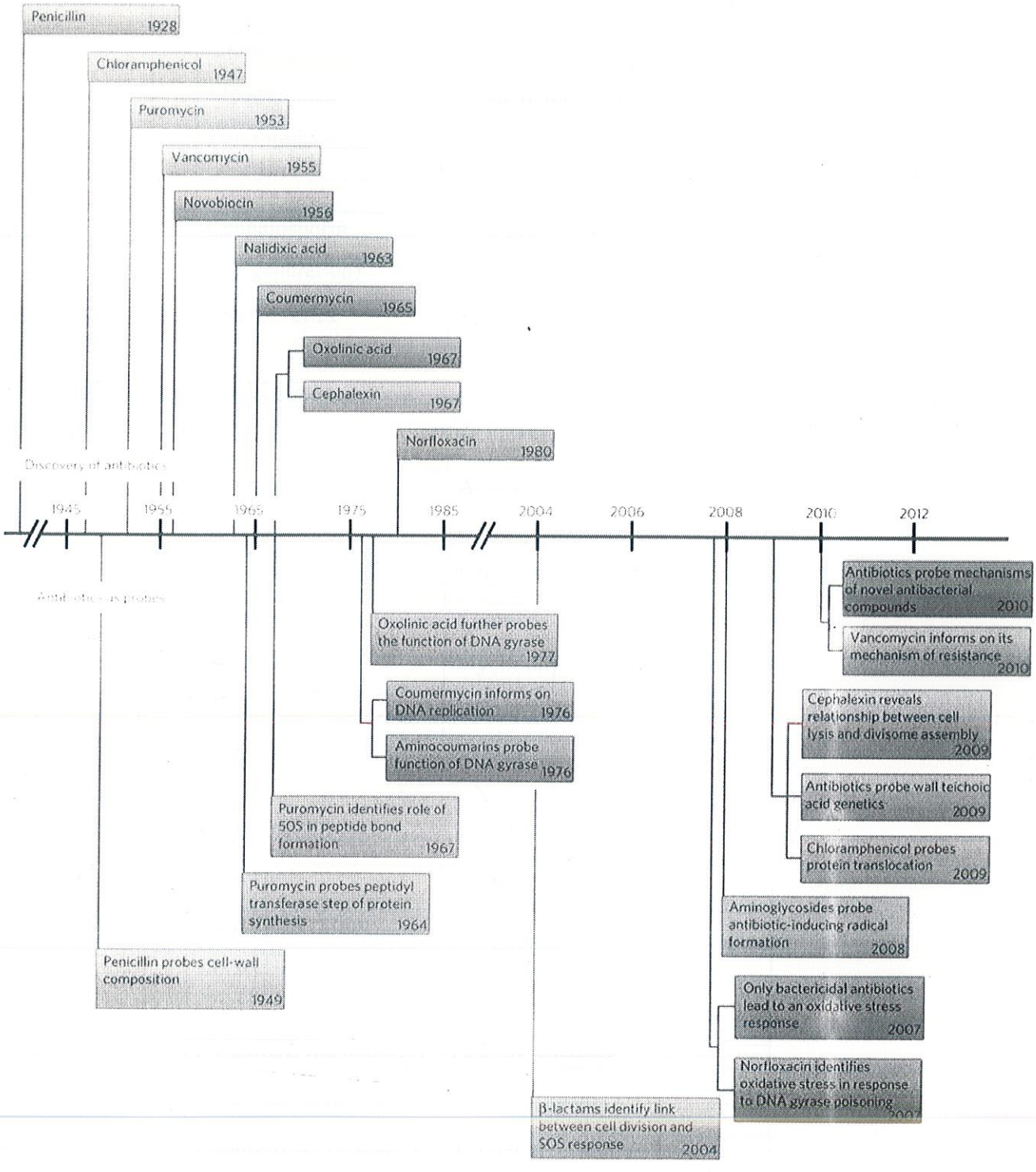
MECHANISM OF ACTION

Beta-lactam antibiotics inhibit the growth of sensitive bacteria by inactivating enzymes located in the bacterial cell membrane, which are involved in the third stage of cell wall synthesis. Beta-lactams inhibit not just a single enzyme involved in cell wall synthesis, but a family of related enzymes (four to eight in different bacteria), each involved in different aspects of cell wall synthesis. These enzymes can be detected by their covalent binding of radioactively-labeled penicillin (or other beta-lactams) and hence have been called penicillin binding proteins (PBPs).

History of Antibiotics

Late 1800s	The search for antibiotics began in the late 1800s, with the growing acceptance of the germ theory of disease, a theory which linked bacteria and other microbes to the causation of a variety of ailments. As a result, scientists began to devote time to searching for drugs that would kill these disease-causing bacteria.
1871	The surgeon Joseph Lister, began researching the phenomenon that urine contaminated with mold would not allow the successful growth of bacteria.
1890s	German doctors, Rudolf Emmerich and Oscar Low were the first to make an effective medication that they called pyocyanase from microbes. It was the first antibiotic to be used in hospitals. However, the drug often did not work.
1928	Sir Alexander Fleming observed that colonies of the bacterium Staphylococcus aureus could be destroyed by the mold Penicillium notatum, demonstrating antibacterial properties.
1935	Prontosil, the first sulfa drug, was discovered in 1935 by German chemist Gerhard Domagk (1895–1964).
1942	The manufacturing process for Penicillin G Procaine was invented by Howard Florey (1898–1968) and Ernst Chain (1906–1979). Penicillin could now be sold as a drug. Fleming, Florey, and Chain shared the 1945 Nobel Prize for medicine for their work on penicillin.
1943	In 1943, American microbiologist Selman Waksman (1888–1973) made the drug streptomycin from soil bacteria, the first of a new class of drugs called aminoglycosides. Streptomycin could treat diseases like tuberculosis, however, the side effects were often too severe.
1955	Tetracycline was patented by Lloyd Conover, which became the most prescribed broad spectrum antibiotic in the United States.
1957	Nystatin was patented and used to cure many disfiguring and disabling fungal infections.
1981	SmithKline Beecham patented Amoxicillin or amoxicillin/clavulanate potassium tablets, and first sold the antibiotic in 1998 under the tradenames of Amoxicillin, Amoxil, and Trimox. Amoxicillin is a semisynthetic antibiotic.

Timeline Of The Discovery Of Antibiotics And Their Use As Probes Of Bacterial Complexity



Lists Of Antibiotics

New Antibiotics

Class	Generic/Brand
Lipopeptides	Daptomycin (Cubicin)
Fluoroquinolone	Gemifloxacin (Factive)
Lipoglycopeptides	Telavancin (Vibativ), 2009
Cephalosporin (5th generation)	Ceftaroline (Teflaro), 2010
Macrocyclics	Fidaxomicin (Dificid), 2011

Aminoglycoside Antibiotics

Generic	Brand Name
Amikacin	Amikin
Gentamicin	Garamycin, G-Mycin, Jenamicin
Kanamycin	Kantrex
Neomycin	Mycifradin, Myciguent
Netilmicin	Netromycin
Tobramycin	Nebcin

Cephalosporins

Generic	Brand Name
First Generation	
Cefacetrile (cephacetrile)	Celospor, Celtol, Cristacef
Cefadroxil (cefadroxyl)	Duricef, Ultracef
Cefalexin (cephalexin)	Keflex, Keftab
Cefaloglycin (cephaloglycin)	Kefglycin

Cefalotin (cephalothin)	Keflin
Cefazolin (cephazolin)	Ancef, Kefzol
Second Generation	
Cefaclor	Ceclor, Ceclor CD, Distaclor
Cefamandole	Mandol
Cefonicid	Monocid
Cefotetan	Cefotan
Cefoxitin	Mefoxin
Cefprozil (cefproxil)	Cefzil
Cefuroxime	Ceftin, Kefurox, Zinacef
Third Generation	
Cefdinir	Omnicef, Cefdiel
Cefditoren	Spectracef
Cefixime	Suprax
Cefmenoxime	Cefmax
Cefotaxime	Claforan
Cefpodoxime	Vantin
Fourth Generation	
Cefclidine	
Cefepime	Maxipime
Ceftuprenam	
Cefozopran	
Cefpirome	Cefrom
Cefquinome	

Carbapenems

Generic	**Brand Name**
Imipenem,Imipenem/cilastatin	Primaxin
Doripenem	Doribax

Meropenem	Merrem
Ertapenem	Invanz

Quinolone Antibiotics

Generic	Brand Name
First Generation	
Flumequine	Flubactin
Nalidixic acid	NegGam, Wintomylon
Oxolinic acid	Uroxin
Piromidic acid	Panacid
Pipemidic acid	Dolcol
Rosoxacin	Eradacil
Second Generation	
Ciprofloxacin	Cipro, Cipro XR, Ciprobay, Ciproxin
Enoxacin	Enroxil, Penetrex
Lomefloxacin	Maxaquin
Nadifloxacin	Acuatim, Nadoxin, Nadixa
Norfloxacin	Lexinor, Noroxin, Quinabic, Janacin
Ofloxacin	Floxin, Oxaldin, Tarivid
Pefloxacin	Peflacine
Rufloxacin	Uroflox
Third Generation	
Balofloxacin	Baloxin
Gatifloxacin	Tequin, Zymar
Grepafloxacin	Raxar
Levofloxacin	Cravit, Levaquin
Moxifloxacin	Avelox, Vigamox
Pazufloxacin	Pasil, Pazucross
Fourth Generation	

Besifloxacin	Besivance
Clinafloxacin	
Gemifloxacin	Factive
Sitafloxacin	Gracevit
Trovafloxacin	Trovan
Prulifloxacin	Quisnon

Macrolide Antibiotics

Generic	**Brand Name**
Azithromycin	Zithromax
Erythromycin	
Clarithromycin	Biaxin
Dirithromycin	Dynabac
Roxithromycin	Rulid, Surlid
Ketolides	
Telithromycin	Ketek

Penicillins

Generic	**Brand Name**
Amoxicillin	Amoxil, Polymox, Trimox, Wymox
Ampicillin	Omnipen, Polycillin, Polycillin-N, Principen
Bacampicillin	Spectrobid
Carbenicillin	Geocillin, Geopen
Mezlocillin	Mezlin
Penicillin G	Bicillin L-A,Crysticillin 300 A.S., Pentids
Penicillin V	Beepen-VK, Betapen-VK,
Piperacillin	Pipracil

Pivampicillin	
Pivmecillinam	
Ticarcillin	Ticar

Sulfonamides

Generic	**Brand Name**
Sulfamethizole	Thiosulfil Forte
Sulfamethoxazole	Gantanol, Urobak
Sulfisoxazole	Gantrisin
Trimethoprim-Sulfamethoxazole	Bactrim, Bactrim DS, Cotrim, Cotrim DS

Tetracycline Antibiotics

Generic	**Brand Name**
Demeclocycline	Declomycin
Doxycycline	Doryx, Vibramycin
Minocycline	Dynacin, Minocin, Monodox
Oxytetracycline	Terramycin
Tetracycline	Achromycin
Glycylcyclines	
Tigecycline	Tygacil

Peudomonas aeruginosa

It is a Gram-negative, aerobic, coccobacillus bacterium with unipolar motility. *P. aeruginosa* is often preliminarily identified by its pearlescent appearance and grape-like or tortilla-like odour invitro . Definitive clinical identification of *P. aeruginosa* often includes identifying the production of both pyocyanin and fluorescein (pigments), as well as its ability to grow at 42°C. *P. aeruginosa* is capable of growth in diesel and jet fuel, where it is known as a hydrocarbon-using microorganism (or "HUM bug"), causing microbial corrosion. It creates dark, gellish mats sometimes improperly called "algae" because of their appearance.

Although classified as an aerobic organism *P. aeruginosa* is considered by many as a facultative anaerobe, as it is well adapted to proliferate in conditions of partial or total oxygen depletion. This organism can achieve anaerobic growth with nitrate as a terminal electron acceptor, and, in its absence, it is also able to ferment arginine by substrate level. Adaptation to microaerobic or anaerobic environments is essential for certain lifestyles of *P. aeruginosa*, for example, during lung infection in cystic fibrosis patients, where thick layers of lung mucus and alginate surrounding mucoid bacterial cells can limit the diffusion of oxygen.

Pathogenesis

An oppurtunistic, noscomical pathogen of immune compromised individuals, *P. aeruginosa* typically infects the pulmonary tract, urinary tract, burns, wounds, and also causes other blood infections.

Infections	**Details and common associations**	**High-risk groups**
Pneumonia	Diffuse bronchopneumonia	Cystic fibrosis patients
Septic shock	Associated with a purple-black skin lesion ecthyma gangrenosum	Neutropenic patients
Urinary tract infection	Urinary tract catheterization	
Gastrointestinal infection	Necrotising enterocolitis (NEC)	Premature infants and neutropenic cancer patients
Skin and soft tissue infections	Hemorrhage and necrosis	Burns victims and patients with wound infections

It is the most common cause of infections of burn injuries and of the outer ear (otitis externa), and is the most frequent colonizer of medical devices (e.g., catheters). *Pseudomonas* can, in rare circumstances, cause community-acquired pneumonias, as well as ventilator-associated pneumonias, being one of the most common agents isolated in several studies. Cystic fibrosis patients are also predis-

posed to *P. aeruginosa* infection of the lungs. The most common cause of burn infections is *P. aeruginosa*. *P. aeruginosa* is frequently associated with osteomyelitis involving puncture wounds of the foot, believed to result from direct inoculation with *P. aeruginosa* via the foam padding found in tennis shoes, with diabetic patients at a higher risk.

Treatment

P. aeruginosa is frequently isolated from nonsterile sites (mouth swabs, sputum, etc.), and, under these circumstances, it often represents colonization and not infection. The isolation of *P. aeruginosa* from nonsterile specimens should, therefore, be interpreted cautiously, and the advice of a microbiologist or infectious diseases physician/pharmacist should be sought prior to starting treatment. Often no treatment is needed.

Phage therapy against *P. aeruginosa* remains one of the most effective treatments, which can be combined with antibiotics, has no contraindications and minimal adverse effects. Phages are produced as sterile liquid, suitable for intake, applications etc. Antibiotics that have activity against *P. aeruginosa* may include:

- aminoglycosides (gentamicin, amikacin, tobramycin, but *not* kanamycin)
- quinolones (ciprofloxacin, levofloxacin, but not moxifloxacin)
- cephalosporins (ceftazidime, cefepime, cefoperazone, cefpirome, ceftobiprole, but *not* cefuroxime, ceftriaxone, cefotaxime)
- antipseudomonal penicillins: carboxypenicillins (carbenicillin and ticarcillin), and ureidopenicillins (mezlocillin, azlocillin, and piperacillin). *P. aeruginosa* is intrinsically resistant to all other penicillins.
- carbapenems (meropenem, imipenem, doripenem, but *not* ertapenem)
- polymyxins (polymyxin B and colistin)
- monobactams (aztreonam)

Staphylococcus aureus

Staphylococcus aureus was first identified in pus from a surgical abscess in a knee joint. It is frequently found in the human respiratory tract and on the skin. Although *S. aureus* is not always pathogenic, it is a common cause of skin infections (e.g. boils), respiratory disease (e.g. sinusitis), and food poisoning. The emergence of antibiotic-resistant forms of pathogenic *S. aureus* (e.g. MRSA) is a worldwide problem in clinical medicine.

S. aureus causes illnesses like minor skin infections, such as pimples, impetigo, boils (furuncles), cellulitis folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), bacteremia, and sepsis. Its incidence ranges from skin, soft tissue, respiratory, bone, joint, endovascular to wound infections. It is still one of the five most common causes of nosocomial infections and is often the cause of postsurgical wound infections.

Treatment and antibiotic resistance

The treatment of choice for *S. aureus* infection is penicillin. Combination therapy with gentamicin may be used to treat serious infections, such as endocarditis, but its use is controversial because of the high risk of damage to the kidneys. The duration of treatment depends on the site of infection and on severity.

Methicillin- resistant S.aureus, **(MRSA)**, is known as a greatly feared strains of *S. aureus* which have become resistant to most β-lactam antibiotics.

Mechanisms of antibiotic resistance

Staphylococcal resistance to penicillin is mediated by penicillinase (a form of β-lactamase) production: an enzyme that cleaves the β-lactam ring of the penicillin molecule, rendering the antibiotic ineffective. Penicillinase-resistant β-lactam antibiotics, such as methicillin, nafcillin, oxacillin, cloxacillin, dicloxacillin, and flucloxacillin, are able to resist degradation by staphylococcal penicillinase.

Resistance to methicillin is mediated via the *mec* operon, part of the staphylococcal cassette chromosome mec (SCC*mec*). Resistance is conferred by the *mecA* gene, which codes for an altered penicillin-binding protein (PBP2a or PBP2') that has a lower affinity for binding β-lactams (penicillins, cephalosporins, and carbapenems). This allows for resistance to all β-lactam antibiotics, and obviates their clinical use during MRSA infections. As such, the glycopeptide vancomycin is often deployed against MRSA.

Escherichia coli

Escherichia coli is a Gram-negative, facultative anaerobic, non-sporulating and rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms (endotherms). Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in humans, and are occasionally responsible for product recalls due to food contamination. The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K_2, and by preventing the establishment of pathogenic bacteria within the intestine.

E. coli and related bacteria constitute about 0.1% of gut flora, and fecal–oral transmission is the major route through which pathogenic strains of the bacterium cause disease. Cells are able to survive outside the body for a limited amount of time, which makes them ideal indicator organisms to test environmental samples for fecal contamination.

Optimal growth of *E. coli* occurs at 37 °C (98.6 °F) but some laboratory strains can multiply at temperatures of up to 49 °C (120.2 °F). Growth can be driven by aerobic or anaerobic respiration, using a large variety of redox pairs, including the oxidation of pyruvic acid, formic acid, hydrogen and amino acids, and the reduction of substrates such as oxygen, nitrate, fumarate, dimethyl sulfoxide and trimethylamine N-oxide. Strains that possess flagella are motile. The flagella have a peritrichous arrangement.

Virulent strains of *E. coli* can cause gastroenteritis, urinary tract infections, and neonatal meningitis. In rarer cases, virulent strains are also responsible for hemolytic-uremic syndrome, peritonitis, mastitis, septicemia and Gram-negative pneumonia.

UPEC (uropathogenic *E. coli*) is one of the main causes of urinary tract infections. It is part of the normal flora in the gut and can be introduced many ways. In particular for females, the direction of wiping after defecation (wiping back to front) can lead to fecal contamination of the urogenital orifices. Anal sex can also introduce this bacteria into the male urethra, and in switching from anal to vaginal intercourse the male can also introduce UPEC to the female urogenital system.

Salmonella enterica typhi

The causative agent of typhoid fever, *Salmonella enterica typhi* (*Salmonella typhi*), is an obligate parasite that has no known natural reservoir outside of humans. Little is known about the historical emergence of human *S. typhi* infections.

S. typhi is a multi-organ pathogen that inhabits the lympathic tissues of the small intestine, liver, spleen, and bloodstream of infected humans. It is not known to infect animals and is most common in developing countries with poor sanitary systems and lack of antibiotics.

It is a gram-negative enteric bacillus. It is a motile, facultative anaerobe that is susceptible to various antibiotics. Currently, 107 strains of this organism have been isolated, many containing varying metabolic characteristics, levels of virulence, and multi-drug resistance genes that complicate treatment in areas that resistance is prevalent. Diagnostic identification can be attained by growth on MacConkey and EMB agars, and the bacteria is strictly non-lactose fermenting. It also produces no gas when grown in TSI media, which is used to differentiate it from other *Enterobacteriaceae*.

Typhoid/ Enteric Fever:

Infection of *S. typhi* leads to the development of typhoid, or enteric fever. This disease is characterized by the sudden onset of a sustained and systemic fever, severe headache, nausea, and loss of appetite. Other symptoms include constipation or diarrhea, enlargement of the spleen, possible development of meningitis, and/or general malaise. Untreated typhoid fever cases result in mortality rates ranging from 12-30% while treated cases allow for 99% survival.

Epidemiology:

The encounter of humans to *S. typhi* is made via fecal-oral route from infected individuals to healthy ones. Poor hygiene of patients shedding the organism can lead to secondary infection, as well as consumption of shellfish from polluted bodies of water. The most common source of infection, however, is drinking water tainted by urine and feces of infected individuals. The estimated inoculum size necessary for infection is 100,000 bacteria. Typhoid fever also represents the second most commonly reported laboratory infection.

The entry of this bacterial species into the human body is most commonly achieved by ingestion, with the importance of aerosol transmission unknown. Once ingested, the organisms multiply in the small intestine over the period of 1-3 weeks, breech the intestinal wall, and spread to other organ systems and tissues. The innate host defenses do little to prevent infection due to the inhibition of oxidative lysis and the ability to grow intracellularly after uptake.

Transmission of *S. typhi* has only been shown to occur by fecal-oral route, often from asymptomatic individuals. 2-5% of previously infected individuals become chronic carriers who show no signs of disease, but actively shed viable organisms capable of infecting others.

Salmonella

Salmonella is a genus of rod-shaped, gram- negative, non-spore-forming, predominantly motile enterobacteria with diameters around 0.7 to 1.5 µm, lengths from 2 to 5 µm, and flagella that grade in all directions (i.e., peritrichous). They are chemoorganotrophs, obtaining their energy from oxidation and reduction reactions using organic sources, and are facultative anaerobes. Most species produce hydrogen sulfide, which can readily be detected by growing them on media containing ferrous sulfate, such as TSI. Most isolates exist in two phases: a motile phase I and a nonmotile phase II. Cultures that are nonmotile upon primary culture may be switched to the motile phase using a Cragie tube.]

Salmonella is closely related to the Escherichia genus and are found worldwide in cold- and warm-blooded animals (including humans), and in the environment. They cause illnesses such as typhoid fever, paratyphoid fever, and foodborne illness.

Salmonella as disease-causing agents

Salmonella infections are zoonotic and can be transferred between humans and nonhuman animals. Many infections are due to ingestion of contaminated food. For example, recent FDA studies link Guatemalan cantaloupes with *Salmonella panama*. In speaking of other salmonella serotypes, enteritis *Salmonella* and Salmonella typhoid/paratyphoid *Salmonella*,the latter—because of a special virulence factor an a capsule protein (virulence antigen)—can cause serious illness, such as *Salmonella enterica* subsp. *enterica* serovar Typhi. *Salmonella typhi* is adapted to humans and does not occur in other animals.

Salmonella species are facultative intracellular pathogens that enter cells via macropinosomes.

Enteritis salmonellosis or food poisoning Salmonella

This is a group consisting of potentially every other serotype (over a thousand) of the *Salmonella* bacteria, most of which have never been found in humans. These are encountered in various *Salmonella* species, most having never been linked to a specific host, but can also infect humans. It is therefore a zoonotic disease.

The organism enters through the digestive tract and must be ingested in large numbers to cause disease in healthy adults. Gastric acidity is responsible for the destruction of the majority of ingested bacteria. Bacterial colonies may become trapped in mucous produced in the oesophagus.

Salmonellosis is a disease caused by raw or undercooked food. Infection usually occurs when a person ingests foods that contain a high concentration of the bacteria, similar to a culture medium.

However, infants and young children are much more susceptible to infection, easily achieved by ingesting a small number of bacteria. In infants, contamination through inhalation of bacteria-laden dust is possible. After a short incubation period of a few hours to one day, the bacteria multiply in the intestinal lumen, causing an intestinal inflammation with diarrhea that is often mucopurulent and bloody. In infants, dehydration can cause a state of severe toxicosis. The symptoms are usually mild. Normally, no sepsis occurs, but it can occur exceptionally as a complication in weakened or elderly patients (e.g., Hodgin's disease). Extraintestinal localizations are possible, especially *Salmonella* meningitis in children, osteitis, etc.

Enteritis *Salmonella* (e.g., *Salmonella enterica* subsp. *enterica* serovar *enteritidis*) can cause diarrhea, which usually does not require antibiotic treatment. However, in people at risk such as infants, small children, the elderly, *Salmonella* infections can become very serious, leading to complications. If these are not treated, HIV patients and those with suppressed immunity can become seriously ill. Children with sickle cell anaemia who are infected with *Salmonella* may develop osteomyelitis.

Salmonella bacteria can survive for weeks outside a living body, and they are not destroyed by freezing. Ultraviolet radiation and heat accelerate their demise; they perish after being heated to 55 °C (131 °F) for 90 min, or to 60 °C (140 °F) for 12 min. To protect against *Salmonella* infection, heating food for at least ten minutes at 75 °C (167 °F) is recommended, so the centre of the food reaches this temperature.

Chapter 2

Objectives and Strategy

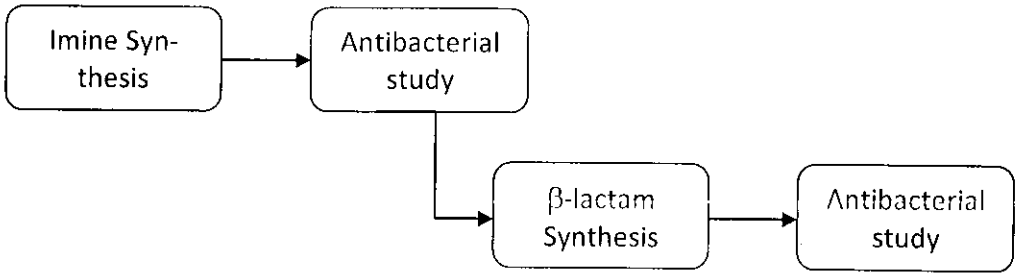
Recent years have witnessed a substantial increase in our understanding of the mechanisms responsible for β-lactam resistance. The structures of the molecular determinants of resistance — particularly in complex with antibiotics or inhibitors — are poised not only to explain resistance, but also to inspire novel methods of combating it. The β-lactam class of antibiotics has proven itself to be invaluable in the treatment of bacterial infections. Bacterial resistance to β-lactam antibiotics can be achieved by any of three strategies: the production of β-lactam-hydrolyzing β-lactamase enzymes, the utilization of β-lactam-insensitive cell wall transpeptidases, and the active expulsion of β-lactam molecules from Gram-negative cells by way of efflux pumps. In recent years, structural biology has contributed significantly to the understanding of these processes and should prove invaluable in the design of drugs to combat β -lactam resistance in the future.

In view of resistant development in bacteria, there is demand to develop new molecules, which should address one or more of following problems:

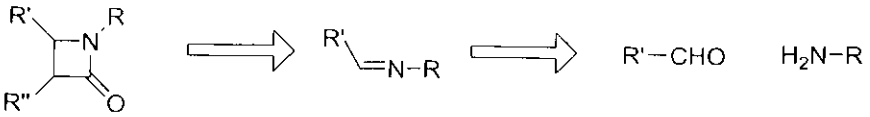
1. New molecules may work as b-lactamase inhibitor
2. New molecule may be unaffected by bacterial defense mechanism
3. New molecule may able to penetrate in to or stay in the cell

Plan/strategy:

We plan to synthesize new β-lactam derivatives, which could act as potential antibiotic or β-lacatamse inhibitors. In present study we have planned only synthesis and antibacterial studies.



A β-lactam could be synthesized from imine, which is a condensation product of aldehyde and amine.



8 3 1 2

Experimental

General method for the preparation of imines[5]

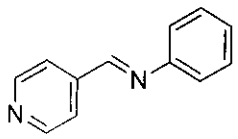
A mixture of 4-pyridine aldehyde **1**(0.001 mol)and amine (0.001 mol) in absolute ethanol was refluxed. The reaction was monitored by TLC. When reaction was completed, the reaction mixture was cooled in ice-water bath, and a drop of conc. sulphuric acid was added. The separated solid was filtered under suction, washed and recrystallized from ethanol.

General method for the preparation of β-lactams[6]

To imine (0.02mol) dissolved in1,2-dichloroethane (10 mL) in round bottom flask fitted with reflux condenser.triethylamine (0.02 mol)was added. The reaction mixture was stirred and heated to 50 °C.Chloroacetylchloride (0.02 mol) in 5ml 1,2-dichloroethane was added slowly with constant stirring . The reaction mixture was allowed to stir at room temperature for 30 minutes and then refluxed till the reaction completes (TLC monitoring).The reaction mixture was cooled and ppt was filtered. Filtrate was extracted with DCM and concentrated. All solids were combined and triturated with water and then with hexane. Solid thus obtained were used without further purification.

Synthesis of *N*-(pyridin-4-ylmethylene)aniline (3A)

A mixture of 4-pyridine aldehyde **1**(0.0186 mol) and aniline**2A** (0.0186mol) in absolute ethanol was refluxed. The reaction was monitored by TLC. When reaction was completed, the reaction mixture was cooled in ice-water bath, and a drop of conc. sulphuric acid was added. The separated solid was filtered under suction, washed and recrystallized from ethanol to yield **3A** as greenish yellow solid (2 g, 59.04%).

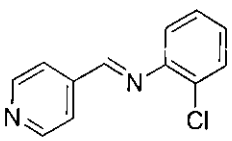


MP:65°C (uncorrected)

Rf :0.58 (Acetone:Toluene :: 1:4)

Synthesis of 2-chloro-N-(pyridin-4-ylmethylene)aniline (3B)

A mixture of 4-pyridine aldehyde **1** (0.02 mol) and 2-chloroaniline **2B**(0.02 mol) in absolute ethanol was refluxed. The reaction was monitored by TLC. When reaction was completed, the reaction mixture was cooled in ice-water



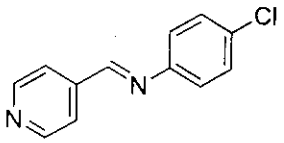
bath, and a drop of conc. sulphuric acid was added. The separated solid was filtered under suction, washed and recrystallized from ethanol to yield **3B** as greenish colour solid (4.13 g, 95.6%).

MP: 75°C (uncorrected)

Rf : 0.44 (Acetone:Toluene :: 1:4)

Synthesis of 4-chloro-N-(pyridin-4-ylmethylene)aniline (3C)

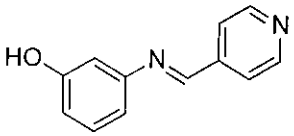
A mixture of 4-pyridine aldehyde **1** (0.02 mol)and 4-chloroaniline**2C**(0.02 mol) in absolute ethanol was refluxed. The reaction was monitored by TLC. When reaction was completed, the reaction mixture was cooled in ice-water bath, and a drop of conc. sulphuric acid was added. The separated solid was filtered under suction, washed and recrystallized from ethanol to yield **3C** as greenish yellow liquid



Rf : 0.48(Acetone:Toluene :: 1:4)

Synthesis of 3-((pyridin-4-ylmethylene)amino)phenol (3D)

A mixture of 4-pyridine aldehyde **1** (0.02 mol)and3-aminophenol(0.02 mol) in absolute ethanol was refluxed. The reaction was monitored by TLC. When reaction was completed, the reaction mixture was cooled in ice-water bath, and a drop of conc. sulphuric acid was added. The separated solid was filtered under suction, washed and recrystallized from ethanol to yield **3D** as brown solid (1.39 g, 35%).

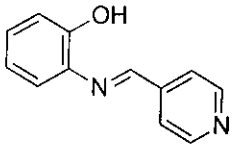


MP:270°C (uncorrected)

Rf :0.42 (Acetone:Toluene :: 1:4)

Synthesis of 2-((pyridin-4-ylmethylene)amino)phenol (3E)

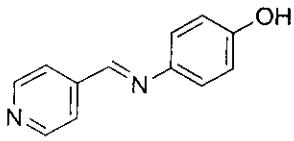
A mixture of 4-pyridine aldehyde **1** (0.02 mol)and2-aminophenol(0.02 mol) in absolute ethanol was refluxed. The reaction was monitored by TLC. When re-action was completed, the reaction mixture was cooled in ice-water bath, and a drop of conc. sulphuric acid was added. The separated solid was filtered under suction, washed and recrystallized from ethanol to yield **3E** as browncrystals (0.37 g, %).



MP:75°C (uncorrected)

Rf :0.52(Acetone:Toluene :: 1:4)

Synthesis of 4-((pyridin-4-ylmethylene)amino)phenol (3F)



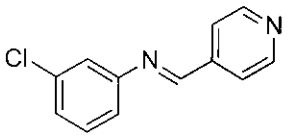
A mixture of 4-pyridine aldehyde **1** (0.02 mol)and4-aminophenol(0.02 mol) in absolute ethanol was refluxed. The reaction was monitored by TLC. When reaction was completed, the reaction mixture was cooled in ice-water bath, and a drop of conc. sulphuric acid was added. The separated solid was filtered under suction, washed and recrystallized from ethanol to yield **3F** as yellow solid (4.3 g, 92%).

MP: 180°C (uncorrected)

Rf : 0.61(Acetone: Toluene :: 1:4)

1H NMR (DMSO-d6, d) 6.82 (2H, m); 7.28 (2H, m); 8.22 (2H, m); 8.64 (1H, s), 8.69 (2H, m); 9.58 (1H, bs).

Synthesis of 3-chloro-N-(pyridin-4-ylmethylene)aniline (3G)

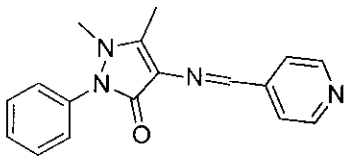


A mixture of 4-pyridine aldehyde **1** (0.02 mol) and 3-chloroaniline(0.02 mol) in absolute ethanol was refluxed. The reaction was monitored by TLC. When reaction was completed, the reaction mixture was cooled in ice-water bath, and a drop of conc. sulphuric acid was added. The separated solid was filtered under suction, washed and recrystallized from ethanol to yield **3G** as greenish yellow solid (3.38 g,78%).

MP: 105°C (uncorrected)

Rf : 0.53 (Acetone:Toluene :: 1:4)

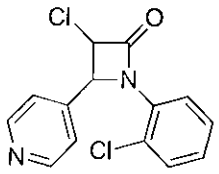
Synthesis of 1,5-dimethyl-2-phenyl-4-((pyridin-4-ylmethylene)amino)-1H-pyrazol-3(2H)-one (3I)



A mixture of 4-pyridine aldehyde **1**(0.0186mol)and 4-aminoantipyrine (0.0186mol) in absolute ethanol was refluxed. The reaction was monitored by TLC. When reaction was completed, the reaction mixture was cooled in ice-water bath, and a drop of conc. sulphuric acid was added. The separated solid was filtered under suction, washed and recrystallized from ethanol to yield **3I** as greenish yellow solid (5.57 g, 95.37%).

MP: 236°C (uncorrected)

Rf : 0.38 (Acetone:Toluene :: 1:4)



4B

Synthesis of 3-chloro-1-(2-chlorophenyl)-4-(pyridin-4-yl)azetidin-2-one (4B)

To2-chloro-N-(pyridin-4-ylmethylene)aniline(0.02mol) dissolved in1,2-

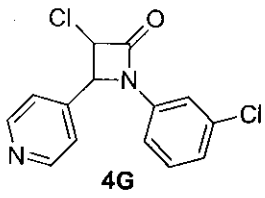
dichloroethane (10 mL) in round bottom flask fitted with reflux condenser,triethylamine(0.02 mol)was added. The reaction mixture was stirred and heated to 50 °C.Chloroacetyl chloride(0.02 mol) in 5ml 1,2-dichloroethane was added slowly with constant stirring . The reaction mixture was allowed to stir at room temperature for 30 minutes and then refluxed till the reaction completes (TLC monitoring).The reaction mixture was cooled and ppt was filtered. Filtrate was extracted with DCM and concentrated. All solids were combined and triturated with water and then with hexane. Solid thus obtained were used without further purification.

Yield: 0.36g, 26.67%

MP: 182°C

Rf: 0.57 (Acetone:Toluene :: 1:4)

Synthesis of 3-chloro-1-(3-chlorophenyl)-4-(pyridin-4-yl)azetidin-2-one (4G)



4G

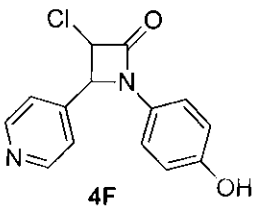
To3-chloro-N-(pyridin-4-ylmethylene)aniline(0.02mol) dissolved in 1,2-dichloroethane (10 mL) in round bottom flask fitted with reflux condenser,triethylamine(0.02 mol)was added. The reaction mixture was stirred and heated to 50 °C.Chloroacetyl chloride(0.02 mol) in 5ml 1,2-dichloroethane was added slowly with constant stirring . The reaction mixture was allowed to stir at room temperature for 30 minutes and then refluxed till the reaction completes (TLC monitoring).The reaction mixture was cooled and ppt was filtered. Filtrate was extracted with DCM and concentrated. All solids were combined and triturated with water and then with hexane. Solid thus obtained were used without further purification.

Yield: 0.42g, 31.11%

MP: 142°C

Rf: 0.18 (Acetone:Toluene :: 1:4)

Synthesis of 3-chloro-1-(4-hydroxyphenyl)-4-(pyridin-4-yl)azetidin-2-one (4F)



4F

To 4-((pyridin-4-ylmethylene)amino)phenol(0.02mol) dissolved in1,2-dichloroethane (10 mL) in round bottom flask fitted with reflux condenser,triethylamine(0.02 mol)was added. The reaction mixture was stirred and heated to 50 °C.Chloroacetylchloride(0.02 mol) in 5ml 1,2-dichloroethane was added slowly with constant stirring . The reaction mixture was allowed to stir at room temperature for 30 minutes and then refluxed till the reaction completes (TLC moni-

toring).The reaction mixture was cooled and ppt was filtered. Filtrate was extracted with DCM and concentrated. All solids were combined and triturated with water and then with hexane. Solid thus obtained were used without further purification.

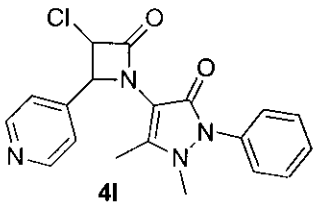
Yield: 0.91g, 71.6%

MP: 185°C

Rf: 0.125 (Acetone:Toluene :: 1:4)

Synthesis of 4-(3-chloro-2-oxo-4-(pyridin-4-yl)azetidin-1-yl)-1,5-dimethyl-2-phenyl-1*H*-pyrazol-3(2*H*)-one (4I)

To 5-dimethyl-2-phenyl-4-((pyridin-4-ylmethylene)amino)-1*H*-pyrazol-3(2*H*)-one(0.004mol) dissolved in1,2-dichloroethane (10 mL) in round bottom flask fitted with reflux condenser,triethylamine(0.02 mol)was added. The reaction mixture was stirred and heated to 50 °C.Chloroacetyl chloride(0.02 mol) in 5ml 1,2-dichloroethane was added slowly with constant stirring . The reaction mixture was allowed to stir at room temperature for 30 minutes and then refluxed till the reaction completes (TLC monitoring).The reaction mixture was cooled and ppt was filtered. Filtrate was extracted with DCM and concentrated. All solids were combined and triturated with water and then with hexane. Solid thus obtained were used without further purification.



4I

Yield: 1.11g, 88 %

MP: 190°C

Rf: 0.24 (Acetone:Toluene :: 1:4)

Antibiotic Susceptibility testing

Species and strains of species of microorganisms have varying degree of susceptibility to different antibiotics. Furthermore, the susceptibility of an organism to a given antibiotic may change, especially during treatment.

Various methods of antibiotic susceptibility testing are:

1. Quantitative Methods
2. Qualitative Methods
3. Automated Susceptibility Tests
4. Newer Non-Automated Susceptibility Tests

5. Molecular Techniques

Quantitative Methods:

In these tests, the minimum amount of antibiotic that inhibits the visible growth of an isolate or MIC is determined. Bacterial isolate is subjected to various dilutions of antibiotics. The highest dilution of antibiotic that has inhibited the growth of bacteria is considered as MIC. These tests can be performed on broth or agar.

1. Broth dilution methods

a. Macrobroth dilution MIC tests

b. Microbroth dilution MIC tests

2. Agar dilution methods

Description and Significance of Microbiology

Microbiology is a specialized area of biology that concerns with the study of microbes ordinarily too small to be seen without magnification. Microorganisms are microscopic and independently living cells that, like humans, live in communities. Microorganisms include a large and diverse group of microscopic organisms that exist as single cell or cell clusters (e.g., bacteria, archaea, fungi, algae, protozoa and helminths) and the viruses, which are microscopic but not cellular. While bacteria and archaea are classed as prokaryotes the fungi, algae, protozoa and helminths are eukaryotes . Microorganisms are present everywhere on earth, which includes humans, animals, plants and other living creatures, soil, water and atmosphere.

BROTH MICRODILUTION(MIC)

Dilution susceptibility testing methods are generally used to determine the minimal concentration of an antimicrobial agent required to inhibit or kill a microorganism. Antimicrobial agents are usually tested atserial dilutions, and the lowest concentration that inhibit visible growth of an organism is regarded as the MIC. The concentration range used may vary with the drug, the organism tested, and the site of the infection. The antimicrobial dilutions contained in wells of a microdilution tray (usually 96 well trays). Obtain result are further reported as the actual MIC or categorically as Susceptible (S), Intermediate (I), or Resistant (R).

*E.coli*become a model organism for studying many of life's essential processesdue to its rapid growth rate and simple nutritional requirements. Researches have well established information about *E. coli*'s genetics and completed many of its genome sequences

Procedure for Antimicrobial Susceptibility Test

1. Prepare 500ml of nutrient broth for maintaining microorganisms, cultivating fastidious organisms
   a. Suspend 13 grams in 1000 ml distilled water. Heat, if necessary, to dissolve the medium completely. Dispense as desired and sterilize by autoclaving at 15 lbspressure(121°C) for 15 minutes.
2. Prepare 500ml of nutrient agar for the cultivation of a wide variety of microorganisms.
   a. Suspend 23 g of the medium in one liter of purified water.
   b. Heat with frequent agitation and boil for one minute to completely dissolve the medium.
   c. Autoclave at 121°C for 15 minutes.
3. Inoculate the medium with isolated colonies or a loopful of pure culture from broth.
4. Streak for isolation.
5. Incubate aerobically at 35°C for 18 – 24 hours or longer if necessary.
6. Isolate single colony of E.coli.Inoculate the medium with the isolated colony.
7. Incubate aerobically at 35°C for 18 – 24 hours or longer if necessary.
8. Dilute the drug with DMSO.
9. Prepare the 96 well plate for the antimicrobial susceptibility test.

CONTROLS

1. Media
2. Media + Culture
3. Media + Drug
4. Drug
5. Solvent (DMSO)

Dilution of drug

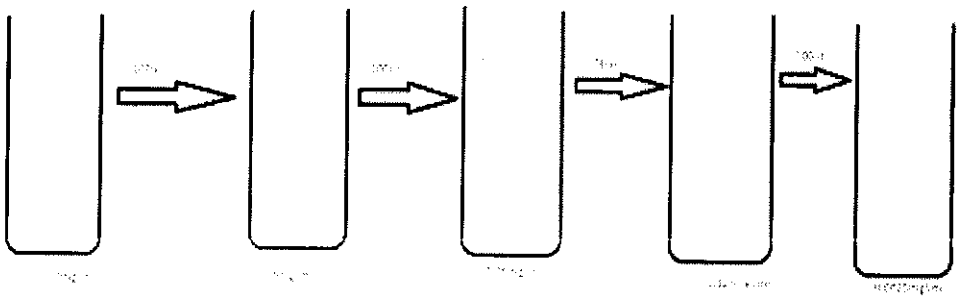


Figure.drug dilation

Procedure for MIC testing:

Microbes tested for antimicrobial activity are as follows:

1. *E. coli* ATCC 25922
2. *S. aureus*ATCC 7447
3. *Salmonella typhimurium*MTCC 98
4. *Pseudomonas aureuginosa* NCTC10662
5. *S. aureus*MTCC 3160
6. *Salmonella typhi*NCTC 786
7. *E. coli* MTCC 723

Preparation of drug solutions:

Appropriate amount of synthesized drug samples were added to the solvent DMSO according to the following table:

Table 1

Drug sample	Molecular Weight	Molarity in mM	Vol in mL	Weight in mg	Vol of 10mM sol
4	182.22	10	1	1.82	5
5	216.67	10	1	2.16	5
7	198.22	10	1	1.98	5
8	198.22	10	1	1.98	5
9	198.22	10	1	1.98	5
10	216.67	10	1	2.16	5
12	292.34	10	1	2.92	5
INH	137.14	10	1	1.37	5

A) Media preparation:

1) 300 mL LB (LeuriaBertani) media was made- 7.5 gms in 300 mL distilled water.
2) 10 mL of media was transferred in 5 test tubes, cotton plugged and autoclaved.

B) Growing of culture:

After autoclaving, inoculation of bacteria was done into a 10 mL test tube and kept for incubation at 37°C overnight.

C) Culture + Media plating- Microbroth dilution

1) Plating of media, culture and drugs were done in 96 wells plate.
2) Plate was labeled, packed and kept in incubation overnight.

D) Procurement of results:

Next day OD of the plate was taken.

Protocol for MBC

Microbes tested for antimicrobial activity are as follows:

1. *E. coli*ATCC 25922
2. *S. aureus*ATCC 7447
3. *Salmonella typhimurium*MTCC 98
4. *Pseudomonas aureuginosa*NCTC10662
5. *S. aureus*MTCC 3160
6. *Salmonella typhi*NCTC 786
7. *E. coli* MTCC 723

Protocol for MBC testing:

A) Preparation of drug solutions:

Appropriate amount of synthesized drug samples were added to the solvent DMSO according to the following table:

Table 2

Drug sample	Molecular Weight	Molarity in mM	Vol in mL	Weight in mg	Vol of 10mM sol
4	182.22	10	1	1.82	5
5	216.67	10	1	2.16	5
7	198.22	10	1	1.98	5
8	198.22	10	1	1.98	5
9	198.22	10	1	1.98	5
10	216.67	10	1	2.16	5
12	292.34	10	1	2.92	5
INH	137.14	10	1	1.37	5

B) Growing of culture

1)Preparation of media-

- for 8 extracts, 50 ml nutrient broth was prepared according to the proportion 13.6/1000ml.
- 2 ml of this media was transferred in 20 test tubes, cotton plugged and autoclaved.

2) Inoculation of microbe – S. aureusATCC 7447.

- 20 µL of the strain was transferred into a test tube containing 2 ml broth.
- this inoculated strain was put into incubator overnight thereby allowing the culture to grow.

3) Streaking :-

- From the previously incubated culture, streaking on agar plate was done.
- Plate was sealed and kept in incubator overnight in order to obtain sinlge colony.

4) Next day, inoculation of single colony into a fresh test tube containing 2 mL broth was done and kept into incubator overnight.

C) Drug + culture plating – micro broth dilution

1) Preparation of drug extracts:-

- To 2 mL broth, 30 µL culture was added and 200 µL of the drug solution was added.
- Similarly 7 extracts were prepared for 7 different drugs.

2) Preparation of culture control:-

- 30 µL of the culture was added to 2 mL broth.

3) Preparation of solvent control:-

- 200 µL of the solvent in which drugs were dissolved which is DMSO is added to 2 mL of the broth media and 30 µL culture was added.

These prepared extracts (drug, solvent and culture) were kept in incubator overnight.

4) Extracts were taken out and kept in 4°C

5) Agar media was prepared, autoclaved and poured into autoclaved petriplates. These plated were wrapped together and kept in incubator for few hours for testing of occurrence of contamination if any.

6) Spreading of extracts on agar plates for obtaining CFU:-

- For every extract 10 serial dilutions of 100 µL were made into 900µL PBS solution.
- From these serially diluted extracts, cfu on agar plates was done.

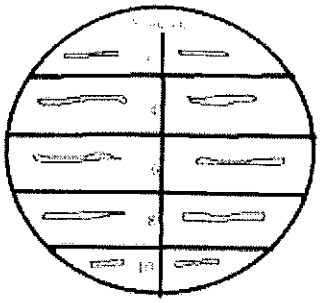


Figure 1

– Plates were sealed properly with paraffin, labeled and kept in incubator overnight.

7) Calculation of CFU:

- Plates were taken out from the incubator.

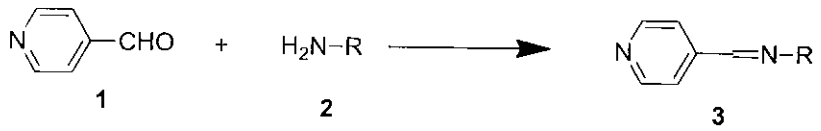
- Colonies in different dilutions were counted and noted.

– CFU was calculated according to the following formula:

$$\text{CFU/mL} = \frac{no.of\ colonies \times dilution\ factor}{amount\ of\ culture\ plated\ in\ mL.}$$

Synthesis of imines

As discussed in strategy and Plan section of this thesis, we first synthesized various imines by condensation of amines with aldehyde as shown in **scheme 1**.



1 2 3

Scheme 1

Mechanism of Imine Formation

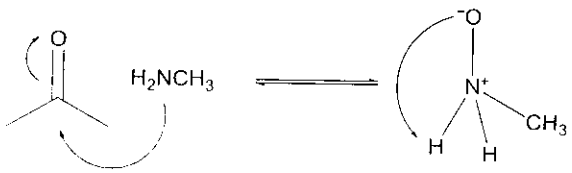


Figure 2

An amine is a stronger nucleophile than an alcohol, so it can add directly to a ketone. This is an acid catalyzed reaction, though. The acid comes in later.

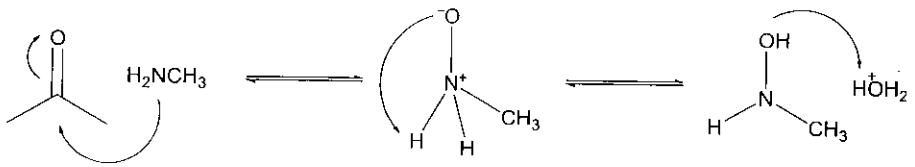


Figure 3

Proton transfer from the nitrogen to the negatively charged oxygen gives a neutral molecule. This is an aminol. This is directly analogoues to the hemiacetal, although the mechanism is slightly different. Formation of the hemiacetal requires an acid catalyst, while the aminol does not. From here on the mechanism will be very similar to acetal formation.

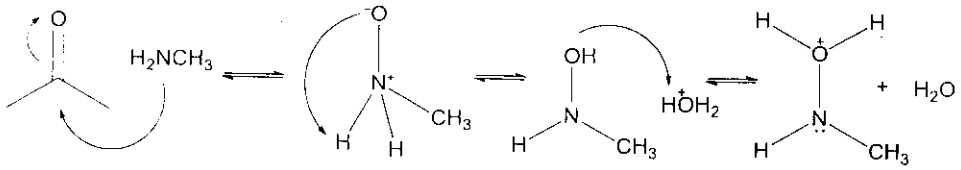


Figure 4

At this point the acid catalyst becomes involved. In order to remove the OH, it must be protonated to give a better leaving group-water.

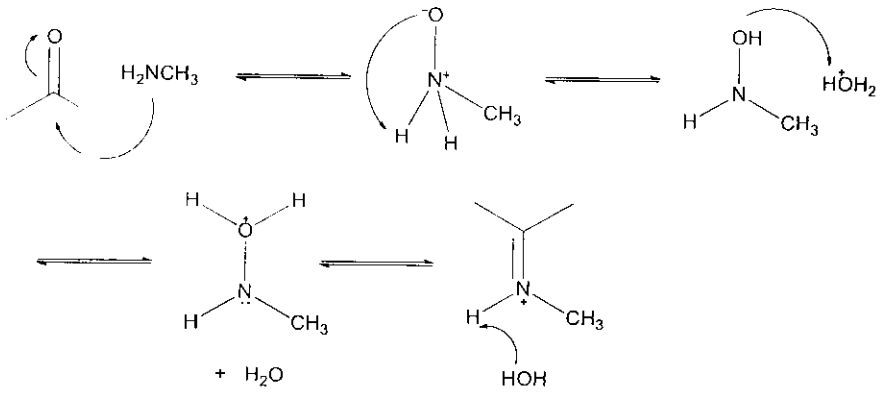


Figure 5

The lone pair on the amine comes down to push out water. This gives protonated imine.

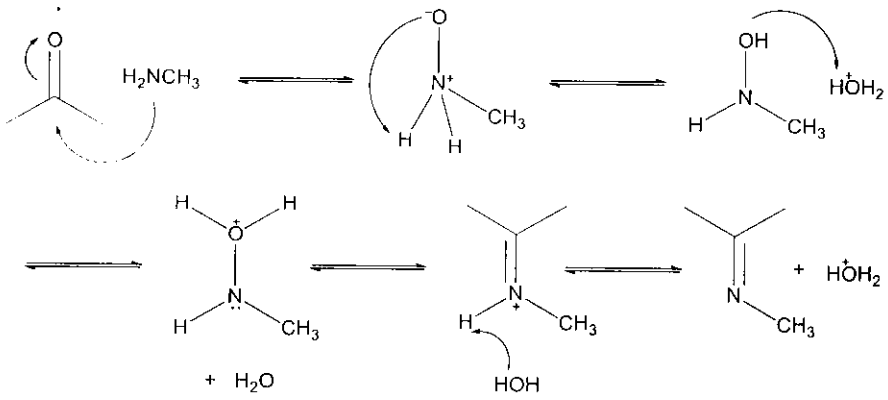


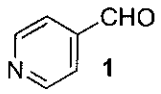
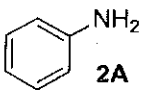
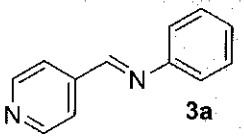
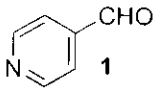
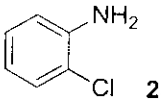
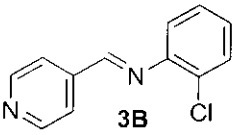
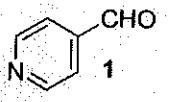
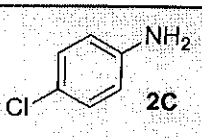
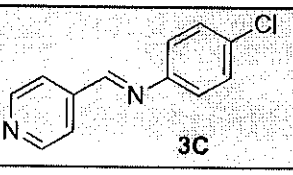
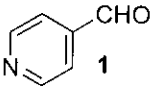
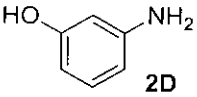
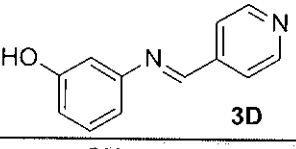
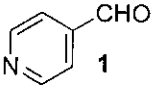
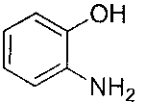
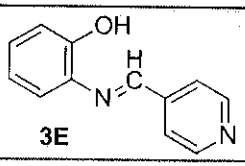
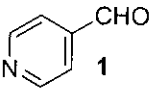
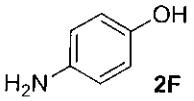
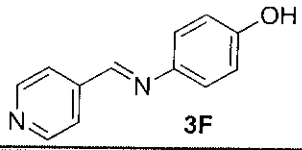
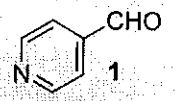
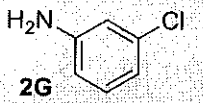
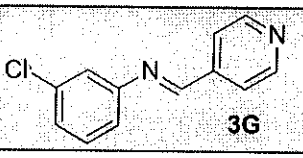
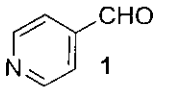
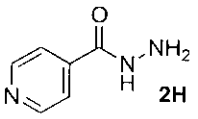
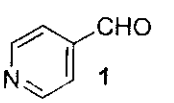
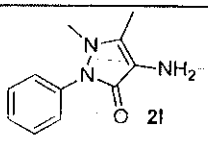
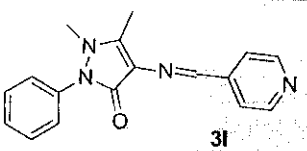
Figure 6

Finally, water accepts the proton from the iminium ion. This is the final step. The acid catalyst is regenerated and a molecule of water is made as well as the imine.

Imines thus formed were washed with water and dried. The product was triturated with hexane and recrystallized with ethanol.Purity of product was checked by TLC.

These results are summarized in table.

Table 3:

SN	Aldehyde	Amine	Imine	Rf / solvent system	MP °C	% yield
1	 1	 2A	 3a	0.58 4:1 tol:acetone	65	59
2	 1	 2B	 3B	0.44 4:1 tol:acetone	75	95.6
3	 1	 2C	 3C	0.48 4:1 tol:acetone	Liq-uid	60
4	 1	 2D	 3D	0.42 4:1 tol:acetone	290	35
5	 1	 2E	 3E	0.52 1:4 Et ac.:hexane	75	93.4
6	 1	 2F	 3F	0.61 4:1 tol:acetone	180	95.14
7	 1	 2G	 3G	0.53 4:1 tol:acetone	135	78.24
8	 1	 2H		Reaction failed		
9	 1	 2I	 3I	0.3 4:1 tol:acetone	236	95.37

Having, imines **2A-I**, we tested these molecules for their susceptibility against some selected strains of bacteria. Based on visual turbidity, drugs in different concentration indicated different activities. The concentration in which a drug showed best activity was selected for further MBC testing. These results are summarized below:

SN	Bacteria	3B		3F		3I		3G		Ampicillin	
1	*E. coli* 723	1000	500	1000	500	1000		1000	500	500	
2	*S. aureus*3160	1000	500	1000		1000		1000	500	500	
3	*P.aureuginosa*	1000		1000		1000		1000	500	500	250
4	*S. typhi*	1000	500	1000		1000		250	125	500	250
5	*S. typhimurium*	1000		1000		1000		250	500	1000	
6	*S. aureus* 7447	1000	500	1000		1000		125	62.5	1000	500
All concentrations are in µg/mL											

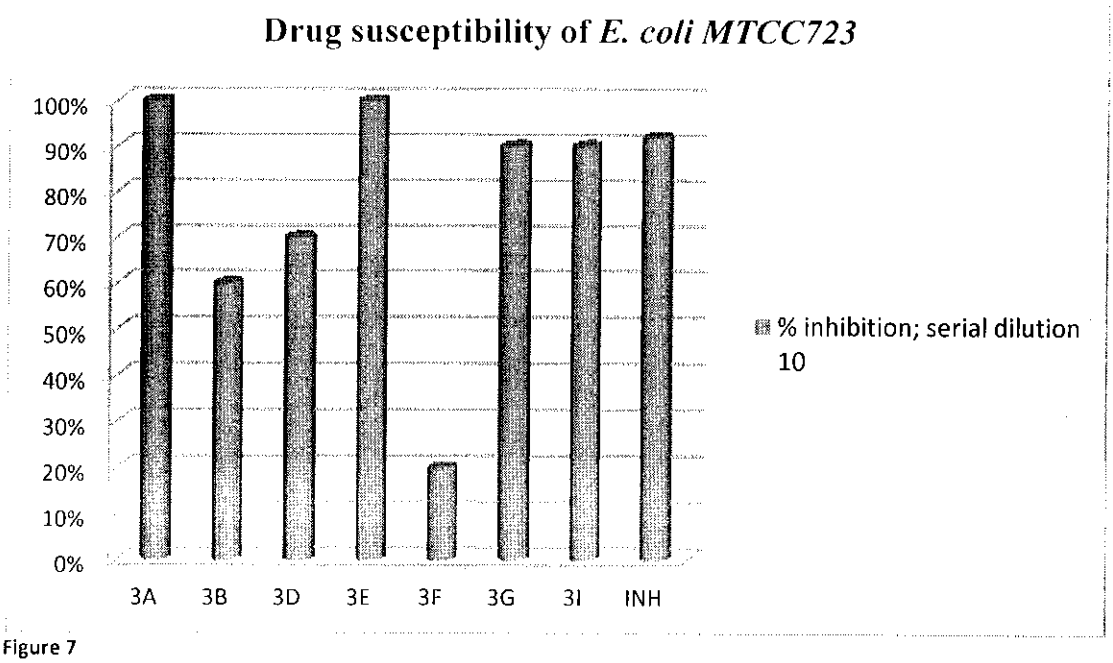


Figure 7

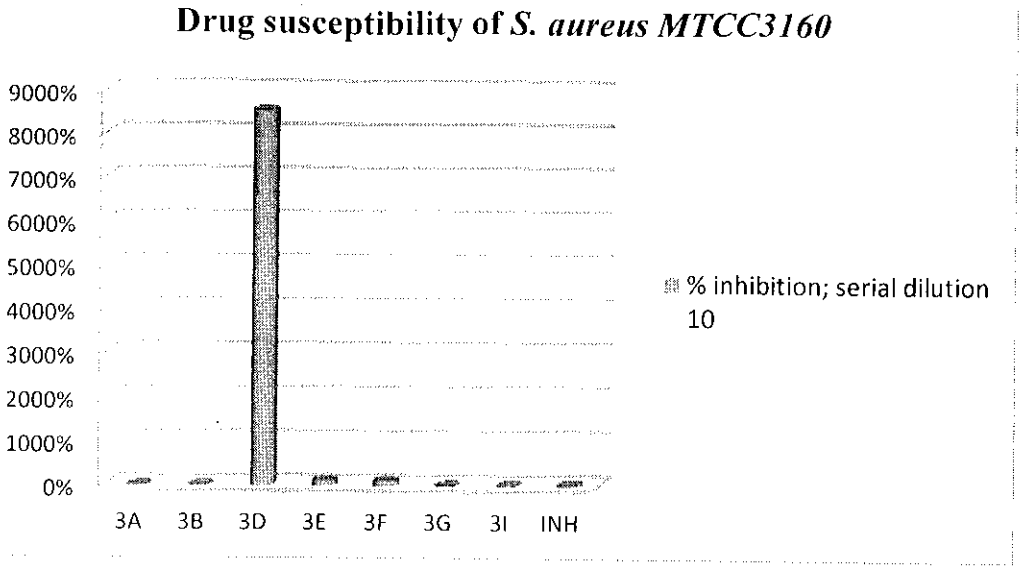
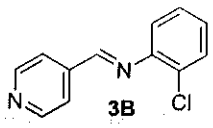
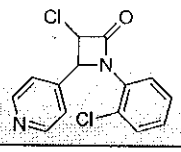
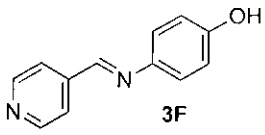
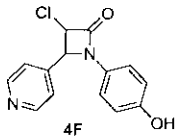
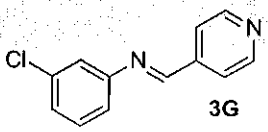
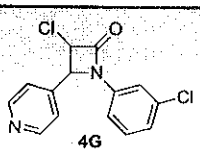
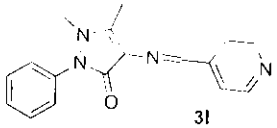
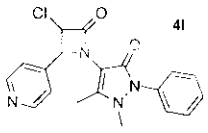


Figure 8

Synthesis of β-lactam derivatives

Having imines and their activity with deferent bacteria, we choose **3B, F, G, I** imines to convert them in β-lactams. These imines were reacted with 2-chloroacetylchloride in presence of triethyl amine. The reaction mechanism of this reaction is shown in figure 9. The experimental results has been summarized in table 4.

Table 4

SN	Imine	β-lactam	Rf / solvent system	MP °C	% yield
I	 3B	 4B	0.57 4:1 tol:acetone	182	26.67
II	 3F	 4F	0.125 4:1 tol:acetone	185	71.6
III	 3G	 4G	0.18 4:1 tol:acetone	142	31.11
IV	 3I	 4I	0.24 4:1 tol:acetone	190	88.09

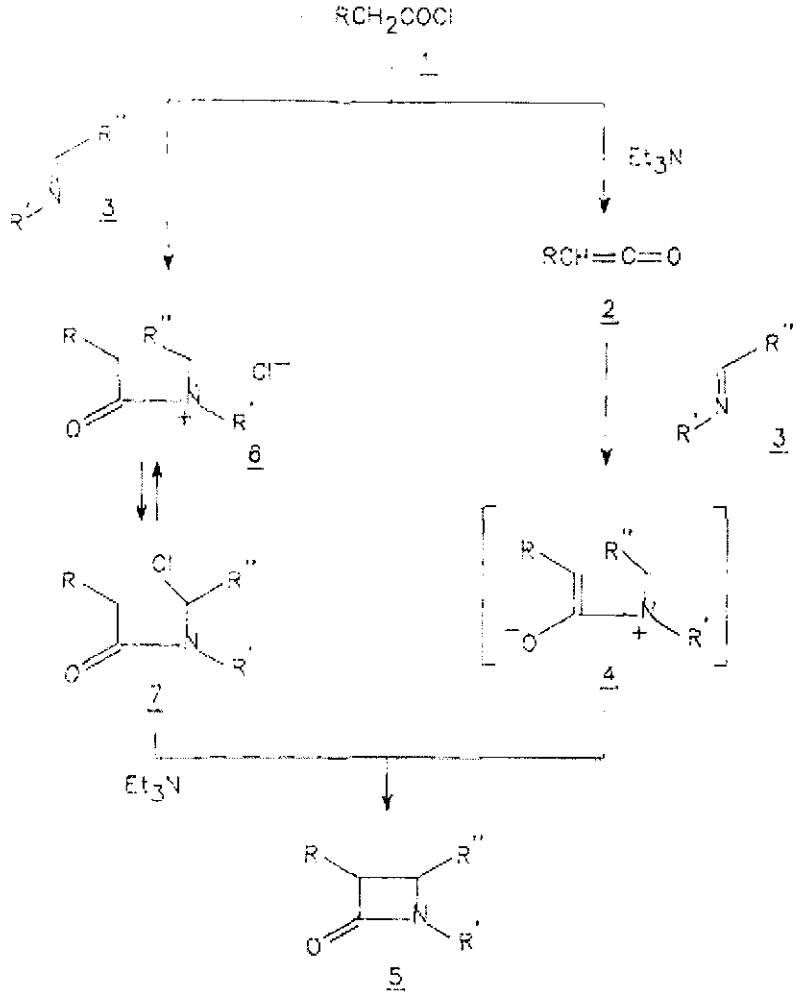


Figure 9 Mechanism of b-lactam formation

These β-lactams were tested for minimum bactericidal concentration. These results has been summarized in following diagrams.

Drug susceptibility of *Salmonella typhi NCTC786*

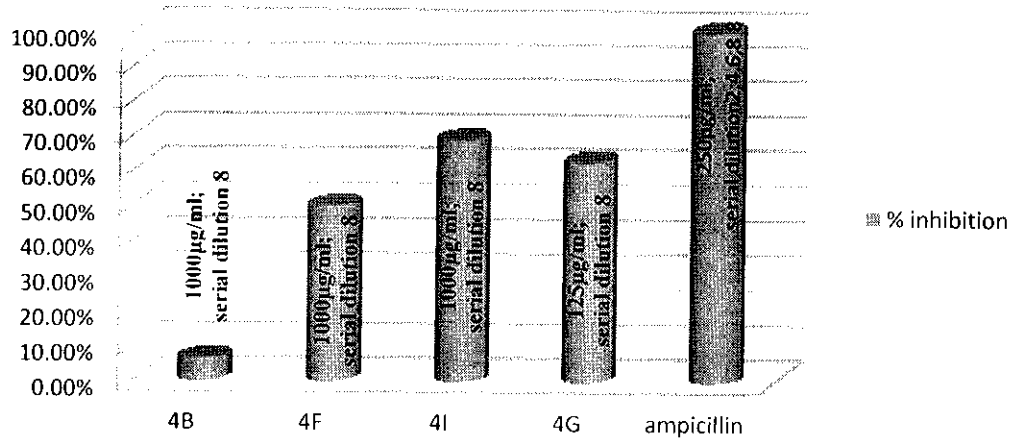


Figure 10

Drug susceptibility of *S. typhimurium MTCC98*

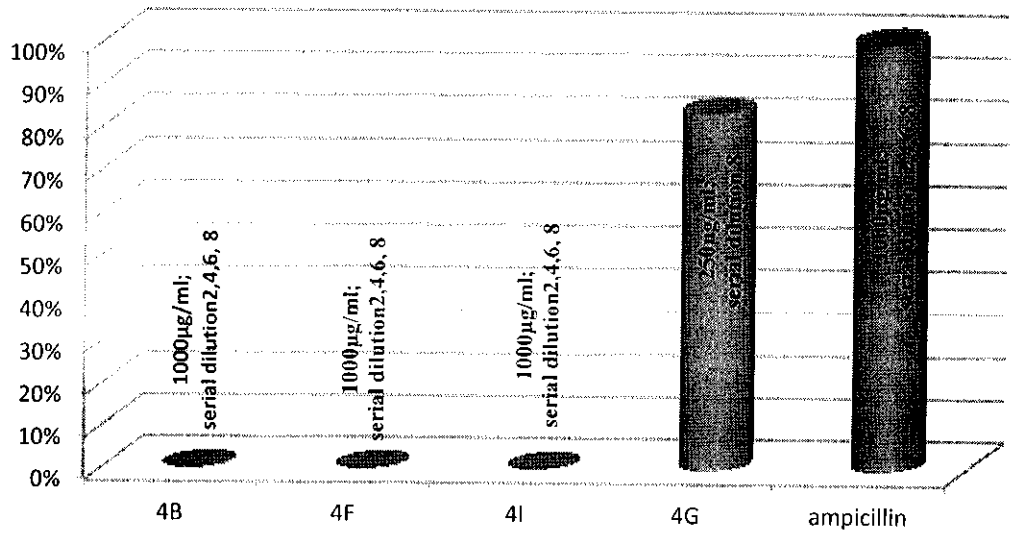


Figure 11

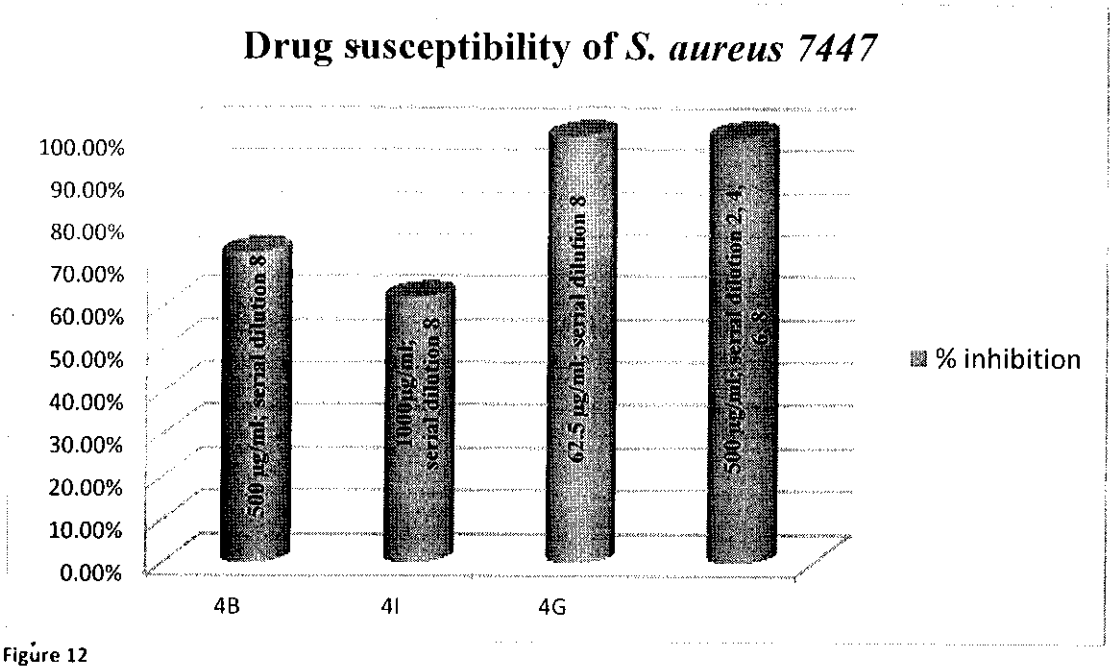


Figure 12

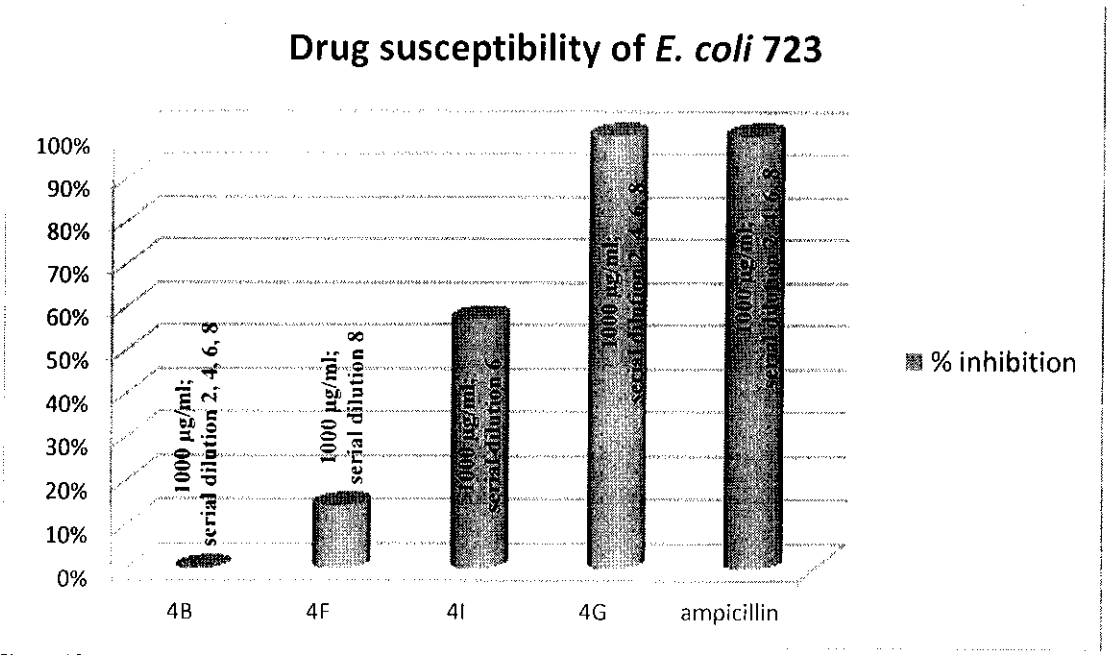


Figure 13

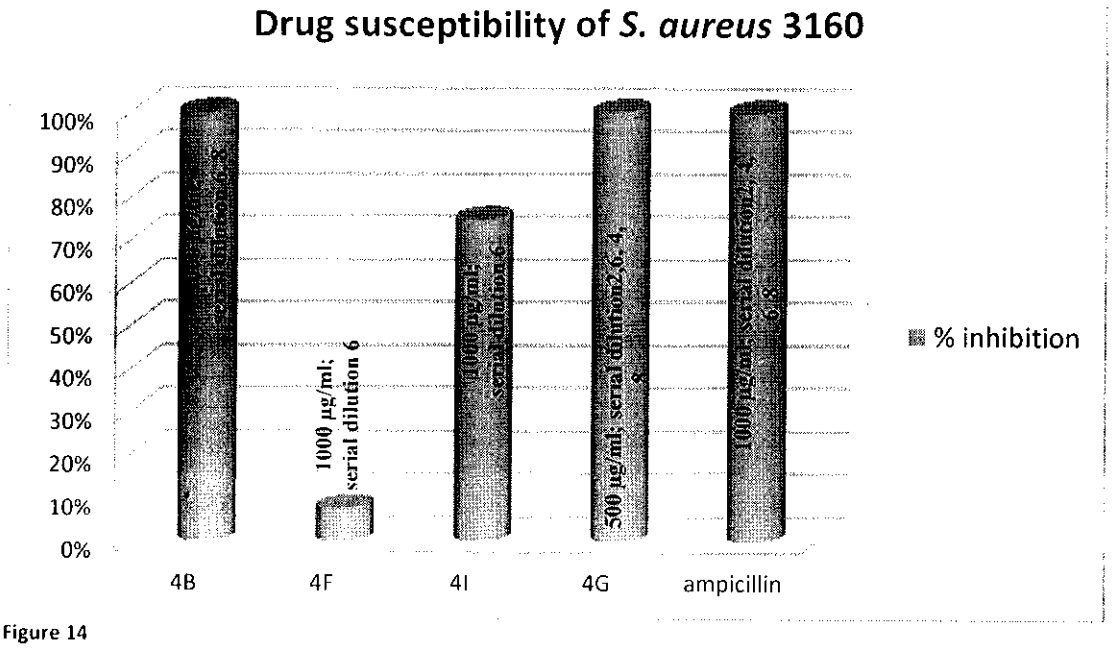

Drug susceptibility of S. aureus 3160
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1000 µg/ml; serial dilution 6
1000 µg/ml; serial dilution 6
500 µg/ml; serial dilution 2^-4
1000 µg/ml; serial dilution 2^-4
4B
4F
4I
4G
ampicillin
% inhibition

Figure 14

Chapter 5

Conclusions

As discussed in strategy and work plan, imines were synthesized by condensation of equimolar amount of aldehyde and amines in ethanol.β-lactam derivatives were prepared by refluxing various imines with 3-chloroacetyl chloride in presence of triethylamine. Reactions were monitored by thin layer chromatography. Purification was carried out either by recrystallization in suitable solvent or by triturating with suitable solvent. The chemical compounds were further taken for microbial testing by broth micro dilution method and data was recorded. These chemical compounds can be further modified and worked upon and can be tested for antimicrobial activity as potential antimicrobial agents.

In conclusion, we have prepared a number of imines and MIC and MBC of these imines were determined. Based on these results some imines were converted to β-lactams and their MBC were determined against pathogenic bacterial strains.

These results show that core structure has a potential as anti-resistance drug. The laboratory will further explore in this direction.

References:

1 Coates, A.; Bax, R. *Nature*. **2002**, 1, 895-907

2 http://dermnetnz.org/treatments/antibiotics.html (accessed July 2011)

3 Williams, D. A. (Ed) *Foyes principles of medicinal chemistry*; 7th ed; Lippincott,2012; pp1073-1125

4 Tripathi.K.D. *Essentials Of Medical Pharmacology*, 6th Ed.; Jaypee Brothers: New Delhi, **2008**; pp 700-717

5 Sethi, A. *Systematic Laboratory Experiment*, 1st ed.; New Age International: New Delhi, **2006**; pp 646

6 Halve, A. K.; Bhadauria, D.; Bhaskar, B.; Dubey, R.; Bhadauria, R., *J Ind Chem Soc*, **2007**, *84*, 193-196

BIODATA OF STUDENTS

Bhavna, born and brought up in Delhi, is pursuing Bachelor of Pharmacy from Jaypee University of Information Technology and will be completing her degree in June 2012. Her interest lies in pharmaceutical chemistry . She would like to contribute similar areas.

ACADEMIC PROFILE

B.pharma ,JUIT	2013	Jaypee University of Information Technology, Waknaghat	*7.4 CGPA*
Class XII,CBSE	2008	Summer Fields School; Gurgaon	*88%*
Class X,CBSE	2006	Summer Fields School; Gurgaon	*92.6%*

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