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DESIGN, SYNTHESIS, CHARACTERIZATION AND IN VITRO KINETIC STUDY OF NOVEL ANTIBACTERIALS PRODRUGS

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ABSTRACT

A number of marketed antibacterial drugs suffer several problems, such as bitter sensation and low stability which lead to patient incompliance. The prodrug approach is considered the most promising and extremely exciting method to overcome such problems. Based on our previously reported density functional theory (DFT) calculations, amoxicillin **ProD 1-2** and cephalexin **ProD 1-2** were designed, synthesized and fully characterized. The intraconversion kinetics for amoxicillin **ProD 1-2** and cephalexin **ProD 1-2** were carried out in different aqueous media and the k_{obs} and $t_{1/2}$ values for the four prodrugs were calculated from a linear regression equation obtained from the plot of log concentration of the residual prodrug versus time. Kinetic studies in 1N HCl, pH 2.5 and pH 5 were selected to examine the intraconversion for the prodrugs to their active parent drugs. The intraconversion of the prodrugs to their active parent drugs was found to be much higher in 1N HCl than in pH 2.5 and pH 5. The experimental $t_{1/2}$ values of amoxicillin **ProD 1** in 1N HCl, pH 2.5 and pH 5 were 2.5, 7 and 81 hours, respectively, and for cephalexin **ProD 1** in 1 N HCl and pH 2.5 were 2 and 14 hours, respectively. On the other hand, the $t_{1/2}$ values of amoxicillin **ProD 2** in 1N HCl and pH 2.5 were 8 and 44 hours, respectively, and for cephalexin **ProD 2** in 1 N HCl was 6 hours. At pH 7.4, the four prodrugs were quite stable and no release of the parent drugs was observed. At pH 5 the hydrolysis of the prodrugs was too slow. In vitro binding test

revealed that the four antibacterial prodrugs were bitterless and it is believed that the lack of the bitter sensation is due to the disability of the prodrugs to interact with the active sites of the tested bitter taste receptors.

KEYWORDS: Prodrugs, bitterness, antibacterials, amoxicillin, cephalexin, enzyme model, DFT calculations, Kirby's N-alkylmaleamic acids.

1. INTRODUCTION

1.1 Background

The palatability of the drug's active ingredient is considered a major obstacle in the development of a patient friendly dosage form. Organoleptic properties, such as odor and taste are crucial factors in the selection of a certain medicine from the available generic medicines available in the pharmaceutical market and having the same active ingredient. The drug's taste is a key issue for doctors and pharmacists when prescribing a medicine for pediatric and geriatric patients. Consequently, the pharmaceutical industries are recognizing the vast importance in masking the unpleasant taste of drugs and a significant number of methods and approaches have been invoked for concealing the objectionable taste of drugs.[1]

A respected number of marketed antibacterial drugs such as amoxicillin, cephalexin, cefuroxime axetil, azithromycin and others suffer various problems and the most striking drawbacks are their low stability in suspension formulation and their bitter sensation which lead to serious patient incompliance. Bitter tastant molecules interact with taste receptors on the tongue to give bitter sensation. Thus, modification on their structural features might provide a solution to overcome this obstacle.

It is believed that amoxicillin's and cephalexin's bitterness is a result of hydrogen bonding between their free amino groups and the amino acids contained in the active sites of the bitter taste receptors.

Therefore, it is quite reasonable to assume that a design and synthesis of prodrugs to replace bitterness antibacterials could increase the patient compliance to antibacterials formulated in solution or syrup dosage forms by reducing or eliminating their bitterness as a result of altering the ability of the antibacterial agent to interact with the active sites of the bitter taste receptors.

It is well known that a large number of therapeutic agents have pharmacological and/or pharmacokinetic barriers, such as low oral absorption, lack of site selectivity, chemical instability, toxicity and poor patient compliance (unpleasant taste, odor, pain at injection site and etc.). The prodrug approach is widely considered among the best approaches to minimize the undesirable properties of a drug while retaining its desirable therapeutic activity. This approach is useful in the optimization of the clinical application of most of the marketed drugs.[2-4]

Prodrugs are bioreversible pharmacologically inactive molecules that prior to exerting the desired pharmacological effect they undergo in vivo enzymatic and/or chemical transformation to their active parent drugs. The prodrug approach is becoming more successful and popular. Nowadays, prodrugs comprise about tenth of the world's marketed medications and fifth of all medications with small molecular weight approved in 2000-2008.[2-4]

1.2 Bitter Taste of Drugs

Several techniques based on physical modifications of active parent drugs have been investigated and revealed to the development of new methods for masking the unpleasant taste of a variety of pharmaceuticals. Some of these techniques include: (1) taste masking with flavors, sweeteners, and amino acids [5] such as the use of monosodium glycyrrhizinate with flavors to mask the bitter taste of guaifenesin, (2) taste masking with lipophilic vehicles such as lipids, lecithin, and lecithin- like substances [6] such as the one composed of magnesium aluminum silicate with soybean lecithin and talampicillin HCl, (3) coating is one of the most efficient and commonly used taste masking techniques. An example of such method is masking the taste of famotidine (a drug for ulcer treatment) by using a combination of water soluble polymers such as polyvinylpyrrolidone, and insoluble polymers, such as cellulose acetate, [7] (4) microencapsulation processes based on the principle of solvent extraction or evaporation, [8] (5) sweeteners are generally used in combination with other taste masking technologies,[9] (6) taste suppressants and potentiators, such as Linguagen's bitter blockers (e.g. adenosine monophosphate): are used for masking the bitter taste of various compounds by competing with binding to the G-protein coupled receptor sites (GPCR): [10] (7) resins are used to mask bitter tastants by forming insoluble resinates through weak ionic bonding with oppositely charged drugs. [11] An example for such technique is the ion exchange resin amberlite which is used to formulate taste masked, fast dissolving, and orally

consumable films of dextromethorphan (a cough suppressant): [12] (8) inclusion complexes**,** the drug molecule fits into the cavity of a complexing agent and forms a stable complex that masks the bitter taste of a drug by decreasing its oral solubility, [13] (9) pH modifiers are capable of generating a specific pH microenvironment in aqueous media that has the ability to facilitate precipitation of the bitter drug compound in saliva thus reducing the overall taste sensation for liquid dosage forms, [14] (10) adsorbates, the compound may be adsorbed or entrapped in the matrix of the adsorbate pore, which may result in a delayed release of the bitter tastant during passage through the oral cavity and mask the taste. [15]

These masking methods have succeeded to overcome or eliminate the unpleasant taste of a number of drugs dosage forms, however, the problem of the bitter sensation of commonly used drugs given to pediatrics and geriatrics still imposes a significant challenge to the health community. Therefore, there is a pressing need to new approaches to be developed for solving this serious problem.

In the past seven years we have developed a novel prodrugs chemical approach which involves the design of prodrugs for drugs having poor bioavailability, low aqueous solubility, inefficient permeation or unpleasant (bitter) taste. This approach is based on intramolecularity using quantum mechanics (QM) such as density functional theory (DFT) and ab initio methods [16] and correlations of experimental and calculated activation energies (reaction's rate). In this approach, no enzyme is involved in the the interconversion of the prodrug to its active parent drug. The release of the active drug from its prodrug is determined and programmed according to the nature (chemical features) of the promoiety bound to the active parent drug. For instance, bitter tastants or drugs interact with the taste receptors located on the tongue to give their bitter taste. Altering the ability and capability of the active drug to interact with the active site/s of the bitter taste receptor/s might have the potential to reduce or completely eliminate its bitterness. This masking can be accomplished by an appropriate modification of the structural features and size of the bitter tastant. Generally, bitter tastant binds to the G-protein coupled receptor-type T2R on the apical membrane of the taste receptor located in the taste buds. [17,18]

It is worth noting that the classic prodrug approach is focused on altering various physiochemical parameters and the interconversion of the prodrug to its active form is usually occur with the involvement of metabolic enzymes such as esterases, amidases, phosphatases or cyctochromes, whereas our novel computational approach, considers a

design of promoieties to be covalently linked to the active drugs and upon reaching the physiological target the prodrug undergoes a controlled intraconversion to a non-toxic moiety and its active parent drug without the need of metabolic enzymes.

1.3 Enzyme Models As Prodrugs Linkers

During the past 60 years a respected number of studies were carried out by several chemists and biochemists for understanding how enzymes catalyze biotransformation reactions.

The findings of these studies revealed that enzyme catalysis is based on the combined effects of the catalysis by functional groups and the ability to reroute intermolecular reactions through alternative pathways by which substrates can bind to preorganized active sites. Enzymatic reactions' rates are in the range of 10^{10} and 10^{18} fold their non-enzymatic bimolecular counterparts. For example, the biotransformation reactions catalyzed by the enzyme cyclophilin are accelerated by10^{5} and those by the enzyme orotidine monophosphate decarboxylase are enhanced by 10^{17}. [19]

Among the scholarly studies that have been devoted to assign the factors playing dominant roles in enzymes catalysis are those done by Bruice, Cohen, Menger and Kirby in which novel enzyme models were invoked and the rate accelerations in such models have been proved to be driven by covalently enforced proximity. The most striking example for such models is the one done by Bruice et al. which involves intramolecular cyclization of dicarboxylic semi esters to yield anhydrides. [20,21] Bruice's study revealed that a relative rate of anhydride formation can reach 5 x 10^{7} upon cyclization of a dicarboxylic semi ester when compared to a similar counterpart's intermolecular reaction. Other examples of enzyme models in which the rate enhancements are due to proximity orientation include: (i) acid-catalyzed cyclization of hydroxy-acids as researched by Cohen et al. [22-24] and Menger, [25-32] (ii) S_N2-based ring-closing reactions as studied by Brown et al. [33] and Mandolini's group, [34] (iii) proton transfers between two oxygens in Kirby's acetals [33-41] and between nitrogen and oxygen in Kirby's enzyme models, [33-41] (iv) proton transfer between two oxygens in rigid systems as reported by Menger, [25-32] and (v) proton transfer between oxygen and carbon in some of Kirby's enol ethers.[33-41] The conclusions emerged from these studies are (a) entropy and enthalpy factors are the driving force for accelerations in rate for intramolecular processes. In ring-closing and proton transfer processes, proximity or/and steric effects were the driving force for rate accelerations, and the enthalpy factor was predominant. (b) The nature of the reaction being intermolecular or intramolecular is dependent on the distance

between the nucleophile and electrophile (the two reacting centers). (c) An effective proton transfer between two oxygens and between nitrogen and oxygen in Kirby's enzyme models were accessible when a strong hydrogen bonding net was formed in the products and the transition states leading to them. [42-64]

Using DFT, ab initio and molecular mechanics methods we have computed the following intramolecular processes (enzyme models): (1) lactonization of dicarboxylic semi-esters to the corresponding anhydrides as explored by Bruice and Pandit, (2) acid-catalyzed cyclization of hydroxy-acids as studied by Cohen et al., (3) proton transfers between two oxygens and between nitrogen and oxygen in Kirby's enzyme models, (4) acid-catalyzed hydrolysis of N-alkylmaleamic acids as researched by Kirby et al. and (5) proton transfer between two oxygens in Menger's rigid hydroxy-acids.

The results emerged from the above mentioned computational studies have been utilized for the design of innovative prodrugs for commonly used drugs containing amine, hydroxyl and phenol functional groups in their structures. [65-98]

For example, unraveling the mechanism of the proton transfer reaction in Kirby's enzyme model (acetals) [99] has revealed to a design and synthesis of several novel prodrugs such as those of aza-nucleosides for the treatment of myelodysplastic syndromes,[82] atovaquone to treat malaria cases [86-88, 100] and statins (simvastatin and atorvastatin) to reduce cholesterol levels in the blood circulation. [83] In these examples, the promoiety of the prodrug was attached to the acetal's hydroxyl group present in the active drug form such that the prodrug chemical device has the potential to undergo chemical cleavage once it reaches the physiological target such as the stomach, intestine, and systemic blood circulation, with rates that are solely determined by the chemical features of the pharmacologically inactive linker (Kirby's enzyme model). Other different linkers such as Kirby's N-alkylmaleamic acids enzyme model [48] was also exploited for the design of a variety of prodrugs such as those of tranexamic and 6-aminocaproic acids for the treatment of bleeding conditions [84] and the antiviral agent acyclovir to treat Herpes Simplex, [85] the anti-hypertensive agent, atenolol [92-93] and the antimalarial agent, atovaquone. [86-88, 100] Another enzyme model which was exploited for the design of dopamine prodrugs for the treatment of Parkinson's disease is Menger's Kemp acid enzyme model. [89] Prodrugs for monoalkyl and dialkyl (monomethyl and dimethyl) fumarates for the treatment of multiple sclerosis and psoriasis were also designed, synthesized and their *in vitro* and *in vivo* kinetics are under investigation. [90]

Furthermore, this approach was also utilized for masking the bitter sensation of the antibacterial agent, cefuroxime axetil, [94] the antihypertensive agent, atenolol, [92-93] and the pain killer agent, paracetamol. [91] The role of the promoiety in cefuroxime axetil, atenolol and paracetamol prodrugs is to block the free amine or the hydroxyl group, which is believed to be responsible for the drug bitter sensation, and to provide a slow release of the active drug from its prodrug. In the case of atenolol and cefuroxime axetil, the difference between the designed prodrugs and their active forms is that the amine group in the active parent drug is replaced with the more stable amide group. It is quite reasonable to assume that replacement of the free amine moiety in the active drugs with an amide group will have the potential to eliminate or reduce significantly the capability of the prodrug to form hydrogen bonding with the amino acids contained in the active site/s of the bitter taste receptors, thus masking the bitter sensation of the parent drug. For example, paracetamol used for alleviating pain and reducing fever has a very strong bitter taste. On the other hand, phenacetin, a pain killer which was withdrawn from the market lacks or has very weak bitter sensation. The chemical difference between both drugs is only in the group in the *para* position of the benzene ring. In the case of paracetamol the *para* position is occupied by a hydroxyl group, whereas in phenacetin the group is ethoxy. Another analgesic and antipyretic drug which was withdrawn from the market due to its toxicity is acetanilide, has a chemical structure similar to that of paracetamol and phenacetin but possess a hydrogen atom in the *para* position of the benzene ring, however, it is quite lack the bitter sensation characteristic of paracetamol. These observations suggest that the hydroxyl moiety on the *para* position of paracetamol is the main contributor for its bitter sensation. It is believed that its bitterness is due to the hydrogen bonding interactions between its hydroxyl and the amino acids located at the active site of the bitter taste receptors. Substituting the phenolic hydroxyl of paracetamol with a chemical group lacking the capability of forming hydrogen bonding with the receptor active site is expected to significantly reduce its binding to the receptor and hence to eliminate its bitterness. In a similar manner, blocking the free amine moiety in atenolol, cefuroxime or any penicillin derivatives containing free amine group such as amoxicillin or cephalexin with a suitable promoiety will have the potential to inhibit the interaction between the prodrug and its bitter taste receptors and hence masks its bitter sensation.

In this manuscript, we have implied this novel prodrug approach in the design of amoxicillin and cephalexin prodrugs using molecular orbital and molecular mechanics methods and correlations between experimental and calculated rate values for Kirby's N-alkylmaleamic

acids process. In this approach, no enzyme is needed to catalyze the conversion of amoxicillin or cephalexin prodrugs to their corresponding active parent drugs.

The use of this approach has the potential to eliminate all disadvantages associated with prodrugs interconversion catalyzed by metabolic enzymes. The *in vivo* interconversion of prodrugs is considered the most vulnerable link in the chain, since there are a variety of intrinsic and extrinsic factors that might affect the process. For instance, the therapeutic profile efficiency of many prodrugs catalyzed by enzymes may be varied due to genetic polymorphisms, age-related physiological changes, or drug interactions, leading to adverse pharmacokinetic, pharmacodynamic, and clinical effects. Furthermore, there are wide interspecies variations in the expression and function of the major metabolic enzymes, and these might pose some obstacles in the preclinical optimization stage.

1.4 Amoxicillin

Amoxicillin is a β-lactam antibiotic (Figure 1): semi-synthetic penicillin which has a moderate-spectrum, used orally to treat infections caused by susceptible microorganisms by which it is susceptible to the action of the β-lactamases. Amoxicillin has a bactericidal action and acts against both Gram positive and Gram-negative bacteria by inhibiting the biosynthesis and repair of the bacterial mucopeptide wall. It is considered the drug of choice within its class since it is well absorbed after oral administration. Amoxicillin is mostly common antibiotics prescribed for children. It has high absorption after oral administration which is not affected by the presence of food. Amoxicillin dose reaches C_{max} about 2 hours after administration and is quickly distributed and eliminated by excretion in urine (about 60%- 75%). The antibacterial effect of amoxicillin is extended by the presence of a benzyl ring in the side chain. Because amoxicillin is susceptible to degradation by β-lactamase-producing bacteria, which are resistant to a broad spectrum of β-lactam antibiotics, such as penicillin, for this reason, it is often combined with clavulanic acid, a β-lactamase inhibitor. This increases effectiveness by reducing its susceptibility to β-lactamase resistance. Amoxicillin has two ionizable groups in the physiological range (the amino group in α-position to the amide carbonyl group and the carboxyl group). Amoxicillin has a good pharmacokinetics profile with bioavailability of 95% if taken orally, its half-life is 61.3 minutes and it is excreted by the renal and less than 30 % is biotransformed in the liver. [101-104]

1.5 Cephalexin

Cephalexin is a first-generation cephalosporin antibiotic (Figure 2): which has been selected as the drug of choice to obtain dosage with improved stability, better palatability and more attractive to pediatrics, cost effective and easy to be administered. Cephalexin is widely used for treatment of skin infections due to its safety profile, and its wide range of activity against both gram positive and gram negative bacteria. Cephalexin is also used for the treatment of articular infections as a rational first-line treatment for cellulitis, it is a useful alternative to penicillins hypersensitivity, and thought to be safe in a patient with penicillin allergy but caution should always be taken, that's because cephalexin and other first-generation cephalosporins are known to have a modest cross-allergy in patients with penicillin hypersensitivity. Cephalexin's mechanism of action is by interfering with the bacteria's cell wall formation, causing it to rupture, and thus killing the bacteria. Cephalexin is zwitterion by which it contains both a basic and an acidic group, its isoelectric point in water is approximately 4.5 to 5. This antibacterial agent has a good pharmacokinetic profile by which it is well absorbed, 80% excreted unchanged in urine within 6 hours of administration. Its half-life is 0.5-1.2 hours and it is excreted *via* the renal. It is used for the treatment of infections including otitis media, streptococcal pharyngitis, bone and joint infections, pneumonia, cellulitis and UTI, and so it may be used to prevent bacterial endocarditis. [105-108]

The major problems in the administration of amoxicillin and cephalexin antibacterial drugs are: (1) the low stability in suspension formulation. These medications are very labile molecules when are exposed to aqueous media. They might undergo hydrolysis when they are standing in solutions. The main cause of their degradation is the reactivity of the strained lactam ring particularly towards hydrolysis, the course of the hydrolysis and the nature of the degradation products are influenced by the pH of the solution. The lactam's carbonyl group in both drugs readily undergoes nucleophilic attack by water and especially hydroxide ion to form the inactive penicilloic acid and (2) their bitter sensation which leads to lack of patient compliance and might create a serious challenge to the pharmacist in pediatrics and geriatrics formulations.

2-Experimental Part

2.1 Calculations Methods

The DFT calculations at B3LYP/6-31G (d,p) and B3LYP/311+G (d,p) levels were carried out using the quantum chemical package Gaussian-2009. [109] The starting geometries of all the

molecules presented in this study were obtained using the Argus Lab program [110] and were initially optimized at the AM1 level of theory, followed by an optimization at the HF/6-31G level. The calculations were carried out based on the restricted Hartree-Fock (RHF) method with full optimization of all geometrical variables. An energy minimum (a stable compound or a reactive intermediate) has no negative vibrational force constant. A transition state is a saddle point which has only one negative vibrational force constant. The "reaction coordinate method" was used to calculate the activation energy in systems **1-9**; [111-112] in this method, one bond length is constrained for the appropriate degree of freedom while all other variables are freely optimized. The activation energy values for the approach processes were calculated from the difference in energies of the global minimum structures and the derived transition states. Similarly, the activation energies of the dissociation processes were calculated from the difference in energies of the global minimum structures and the corresponding transition states. Verification of the desired reactants and products was accomplished using the "intrinsic coordinate method". The transition state structures were verified by their only one negative frequency. Full optimization of the transition states was accomplished after removing any constrains imposed while executing the energy profile. The activation energies obtained from DFT at B3LYP/6-31G (d,p) level of theory for **1-9** were calculated with and without the inclusion of solvent (water). The calculations with the incorporation of a solvent were performed using the integral equation formalism model of the Polarizable Continuum Model (PCM); [113-116] in this model the cavity is created via a series of overlapping spheres. The radii type employed was the United Atom Topological Model on radii optimized for the PBE0/6-31G (d) level of theory.

2.2 Experimental

Inorganic salts were of analytical grade and were used without further purification. Organic buffer components were distilled or recrystallized. Distilled water was redistilled twice before use from all-glass apparatus. Maleic anhydride, succinic anhydride, anhydrous sodium dihydrogen phosphate, sodium lauryl sulfate, triethyl amine, amoxicillin and cephalexin were commercially obtained from Sigma Aldrich. HPLC grade solvents of methanol, acetonitrile and water were purchased from Sigma Aldrich. High purity dichloromethane, ethyl acetate, THF and diethyl ether (> 99%) were purchased from Biolab (Israel). The LC/ESI-MS/MS system used was Agilent 1200 series liquid chromatography coupled with a 6520 accurate mass quadruple-time of flight mass spectrometer (Q-TOF LC/MS). The analysis was

performed in the positive electrospray ionization mode. The capillary voltage was 4.0 kV, the scanned mass range was 200-540 *m/z* (MS).

The high pressure liquid chromatography (HPLC) system consisted of an Alliance 2695 module equipped with 2996 Photodiode array detector from Waters (Germany). Data acquisition and control were carried out using Empower 2 ™ software (Waters, Germany). Analytes were separated on a 4.6 mm x150 mm XBridge® C18 column (5 µm particle size) used in conjunction with a 4.6 x 20 mm, XBridge® C18 guard column. Microfilters of 0.45µm porosity were normally used (Acrodisc® GHP, Waters). pH meter model HM-30G: TOA electronics™ was used in this study to measure the pH value for the buffers. The Sep-Pack C18 6cc (1 g) cartridges were purchased form Waters (Milford, MA, USA). ^{1}H-NMR experiments were performed with a Bruker AvanceII 400 spectrometer equipped with a 5 mm BBO probe. All infrared spectra (FTIR) were obtained from a KBr matrix (4000–400 cm^{-1}) using a PerkinElmer Precisely, Spectrum 100, FT-IR spectrometer.

2.2.1 Preparation of amoxicillin ProD 1-2 (Figure 1)

Amoxicillin **ProD 1:** In a 250 ml round-bottom flask, 2.12 g of amoxicillin trihydrate (5 mmol) was dissolved in 100 ml H_2O, 0.45 g of sodium bicarbonate was added and the resulting solution was stirred for 30 minutes, then 0.5 g of maleic anhydride (5 mmol) was slowly added to the reaction mixture. The pH of the mixture was adjusted to 7. The reaction mixture was stirred at room temperature for 2 hours and the progress of the reaction was monitored by TLC. The reaction's solvent was evaporated using rotary evaporator and the resulting precipitate was washed with ethyl acetate and filtered. The white precipitate formed was dissolved in methanol, filtered, evaporated and dried at 39˚C to yield 2.6 g. M.P. 170 ˚C; ^{1}H-NMR δ (ppm) CD3OD: 1.5 (m, 6H, CH3-C-CH3): 4.2 (d, 1H, J=20 Hz, HN-CH-CH-S,): 4.7 (s, 1H, N-CH-COOH): 5.0 (d, 1H, J=4 Hz, HC-CH-S-C): 5.4 (d, 1H, J=6.4 Hz, NH-CH-C): 5.5 (d, 1H, J=20 Hz, NH-CH-(Ar)): 6.3 (d, 1H, J=10.4 Hz, HOOC-CH=CH-C=O): 6.7 (d, 1H, J=10.4 Hz, HC-CH=C-CH=C): 6.8 (m, 2H, aromatic): 7.3 (m, 2H, aromatic); IR (KBr/ν_{max} cm^{-1}) 1763 (C=O): 1650, 1585, 1369, 1246; *m/z* 486.1 (M+1).

Amoxicillin **ProD 2**: the same procedure was followed as for amoxicillin **ProD 1** but instead of using maleic anhydride, 0.5 g (5 mmol) succinic anhydride was used (product; 2.8 g as a white solid). M.P. 147-150 ˚C; ^{1}H-NMR δ (ppm) CD3OD: 1.5 (m, 6H, CH3-C-CH3): 2.5 (m, 4H, COOH-CH2-CH2-C=O): 3.4 (d, 1H, J=5.6 Hz, HC-CH-S-C): 4.2 (s, 1H, N-CH-COOH):

4.9 (s, 1H, HN-(C=O)-CH-Ar): 5.4 (d, 1H, J=4 Hz, NH-CH-C=O): 6.9 (m, 2H, aromatic): 7.3 (m, 2H, aromatic); IR (KBr/ν_{max} cm^{-1}) 1769 (C=O): 1576, 1514, 1402; *m/z* 488.1 (M+1).

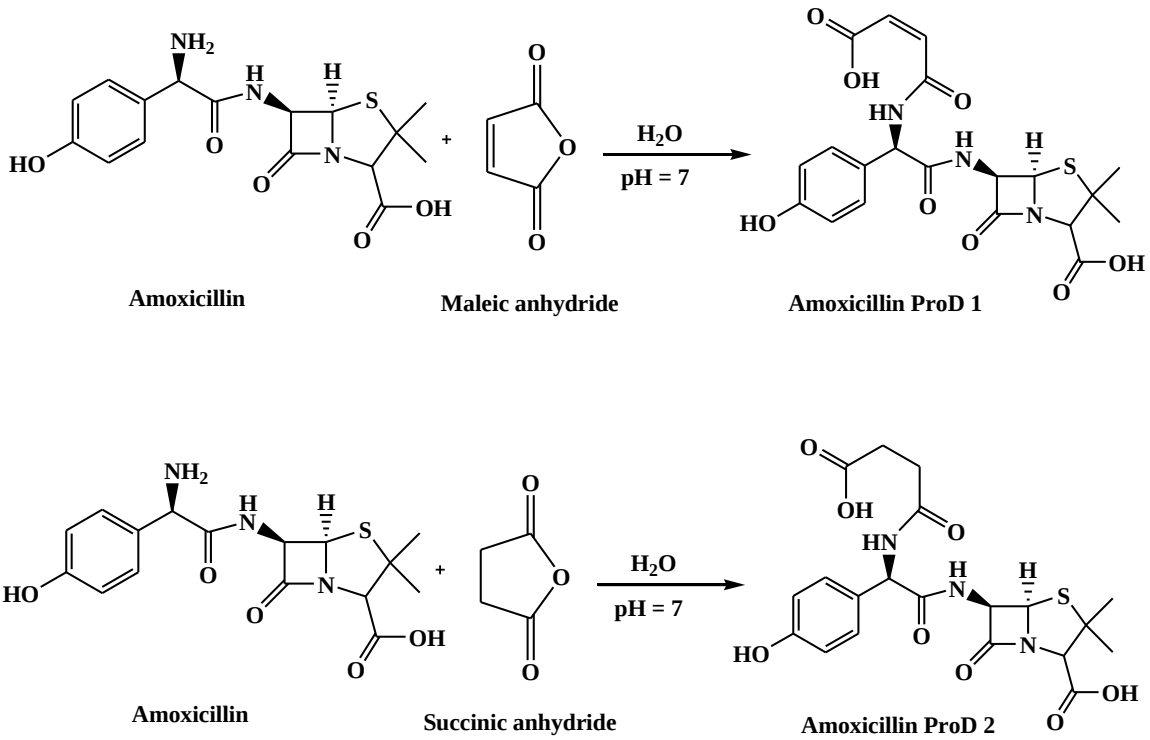


Figure 1: **Schematic representation for the synthesis of amoxicillin ProD 1-2 from its parent drug, amoxicillin, and an anhydride.**

2.2.2 Preparation of cephalexin ProD 1-2 (Figure 2)

Cephalexin **ProD 1**: In a 250 ml round-bottom flask, 1.75 g of cephalexin standard (5 mmol) was dissolved in 100 ml H_2O, 0.45 g of sodium bicarbonate was added and the resulting solution was stirred at room temperature, then 0.52 g of maleic anhydride (5 mmol) was slowly added to the reaction mixture. The pH of the reaction was maintained at pH 7, then was stirred for 2 hours at room temperature and was monitored by TLC. The reaction's solvent was evaporated by rotary evaporator to dryness, and the resulting residue was washed with ethyl acetate. The solid residue obtained was dissolved in 50 ml methanol, dried over $MgSO_4$, filtered and evaporated. The resulting white precipitate was collected and dried at 39˚C to yield 2.9 g. M.P. 156-160 ˚C; ^{1}H-NMR δ (ppm) CD3OD: 1.5 (s, 3H, CH3-C=C): 3.3 (s, 2H, S-CH2-C=C): 4.9 (s, 1H, C-CH-C=O): 4.95 (s, 1H, NH-CH-C=O): 5.5 (d, 1H, J= 12.8 Hz, S-CH-CH-NH): 5.6 (d, 1H, J= 12.8 Hz, NH-CH-CH):): 6.23 (d, 1H, J = 12.8 Hz, HC=CH): 6.30 (d, 1H, J = 12.8 Hz, HC=CH): 7.3-7.5 (m, 5H, aromatic); IR (KBr/ν_{max} cm^{-1}) 1758 (C=O): 1674, 1578, 1249; *m/z* 468 (M+1).

Cephalexin **ProD 2**: the same procedure was followed as for the preparation of cephalexin **ProD 1** but instead of using maleic anhydride, 0.52 g (5 mmol) of succinic anhydride were used (yield; 2.0 g.). M.P. 240 ˚C; ^{1}H-NMR δ (ppm) CD3OD: 2.05 (s, 3H, CH3-C=C): 2.5 (m, 4H, COOH-CH2-CH2-C=O): 3.03 (d, 1H, J=12.6 Hz, NH-CH-CH-N-C): 3.2 (d, 1H, J=12.6 Hz, NH-CH-CH-S, 3.3 (s, 2H, S-CH2-C=C): 4.9 (s, 1H, NH-CH-C=O): 5.5 (s, 1H, Ar-CH-C=O): 7.3-7.6 (m, 5H, aromatic). IR (KBr/ν_{max} cm^{-1}) 1755 (C=O): 1665, 1586, 1255; *m/z* 470 (M+1).

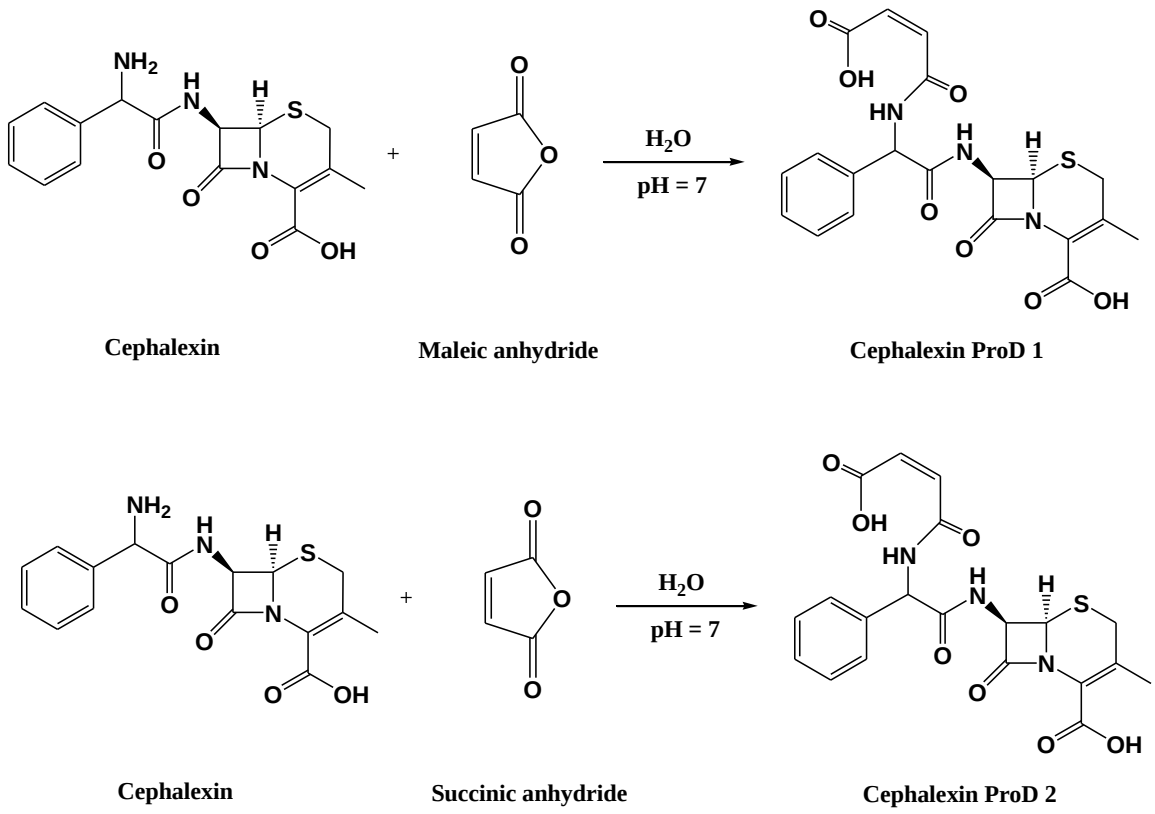


Figure 2: **Schematic representation for the synthesis of cephalexin ProD 1-2 from its parent drug, cephalexin, and an anhydride.**

2.2.3 Kinetic Methods

The intraconversion of 500 ppm amoxicillin **ProD 1-2** solutions, in 1N HCl, buffer pH 2.5, buffer pH 5.0 and buffer pH 7.4, to their parent drug, amoxicillin, was run at 37.0 ˚C and followed by HPLC at a wavelength of 254 nm for amoxicillin **ProD 1** and 230 nm for amoxicillin **ProD 2**.

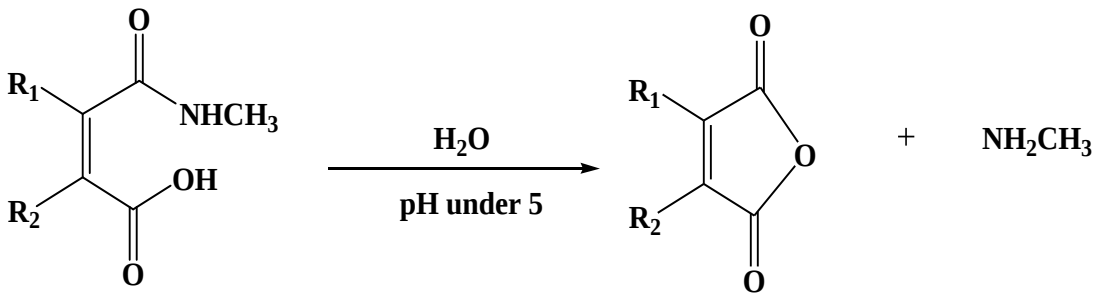
The intraconversion of 500 ppm cephalexin **ProD 1-2** solution, in 1N HCl, buffer pH 2.5, buffer pH 5.0 and buffer pH 7.4, to their parent drug, cephalexin, was run at 37.0 ˚C and followed by HPLC at a wavelength of 230 nm.

3. RESULTS AND DISCUSSION

3.1 Kirby's N-alkylmaleamic acids enzyme model

Kirby et al. have studied the acid-catalyzed hydrolysis of N-alkylmaleamic acids **1-9** (Figure 3) [41] and found that the amide in **1-9** is cleaved due to intramolecular nucleophilic catalysis by the adjacent carboxylic acid group and the rate-limiting step of the reaction is the dissociation of the tetrahedral intermediate. [35-41, 99]

For exploiting Kirby's intramolecular process [35-41, 99] for the design of amoxicillin and cephalexin prodrugs we have explored the mechanism of the intramolecular acid- catalyzed hydrolysis of **1-9** using DFT calculation methods at both B3LYP/6-31G (d,p) and B3LYP/311+G (d,p) levels. The DFT calculations demonstrated that the reaction proceeds by three steps: (i) proton transfer from the carboxylic acid group to the amide carbonyl oxygen, (ii) nucleophilic attack of the carboxylate onto the protonated carbonyl carbon; and (iii) dissociation of the tetrahedral intermediate to yield products. Additionally, the calculations revealed that the efficiency is significantly sensitive to the pattern of substitution on the carbon–carbon double bond. Further, it was found that the hydrolysis rate is linearly correlated with the strain energy of the reaction's tetrahedral intermediate or product. Systems having unstrained tetrahedral intermediates or products are characterized with high rates and vice versa. [42-65]



1; R_1=R_2=H
2; R_1=R_2=Me
3; R_1=H; R_2=Me
4; R_1,R_2 CycIopent-l-ene-1,2-diyl
5; R_1, R_2 Cyclohex-l-ene-1,2-diyl
6; R_1=H; R_2=Et
7; R_1=H; R_2=n-Propyl
8; R_1=H; R_2=Trifluoromethyl
9; R_1=R_2= Trifluoromethyl

Figure 3: Acid-catalyzed hydrolysis of maleamic acids 1-9.

3.2 Hydrolysis studies

Based on the DFT calculations and experimental data for the acid-catalyzed hydrolysis of amide acids **1-9** (Figure 3): [41, 48] four amoxicillin and cephalexin prodrugs were proposed utilizing two different linkers (Figures 4 and 5, respectively**)**. As shown in Figures 4 and 5, the antibacterial prodrugs, amoxicillin **ProD 1-2** and cephalexin **ProD 1-2** are composed of a promoiety containing a carboxylic acid group (hydrophilic moiety) and the rest of the prodrug molecule (a lipophilic moiety).

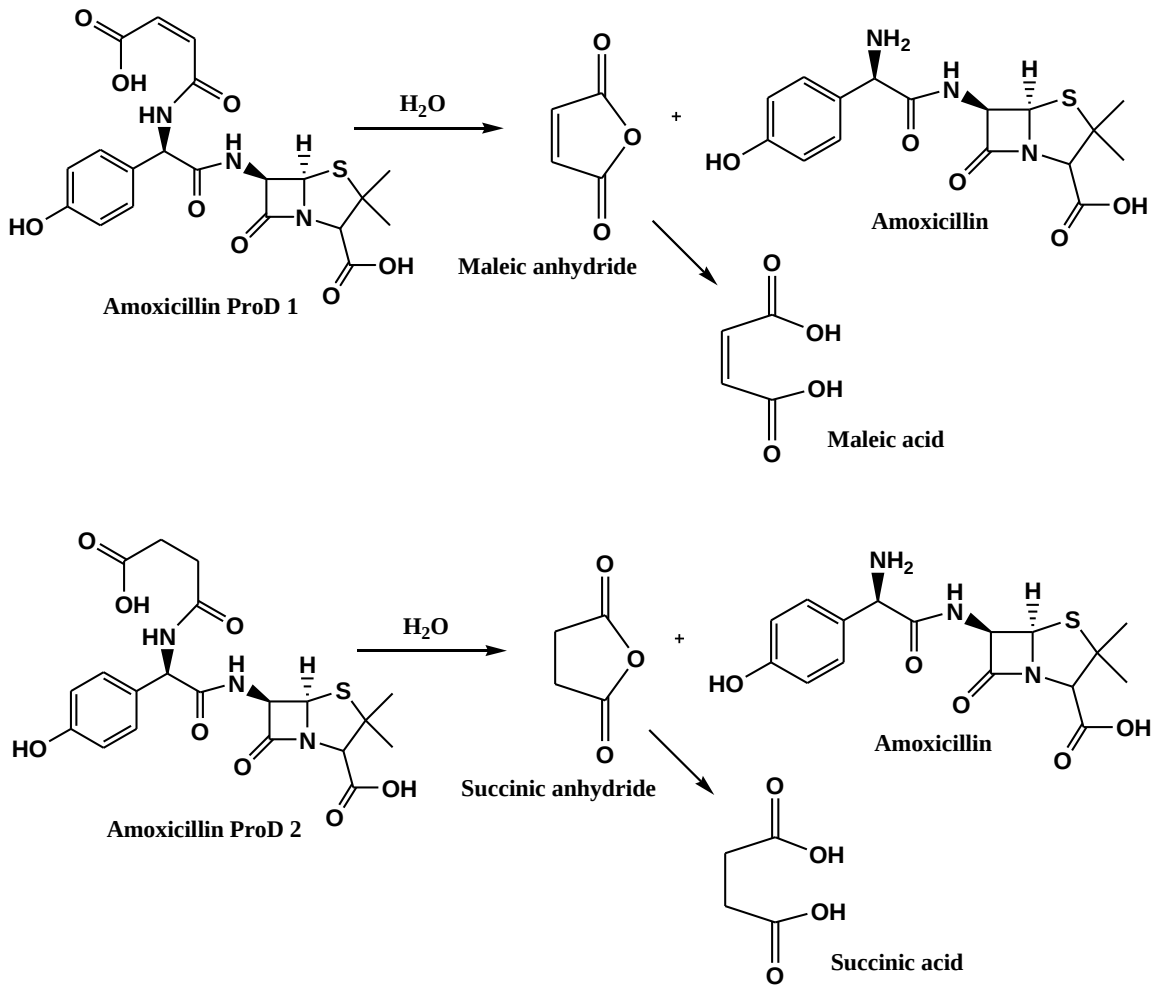


Figure 4: Intraconversion of amoxicillin ProD 1-2 to their parent drug, amoxicillin.

The combination of the hydrophilic and lipophilic groups provides a chemical device with a moderate HLB value that has the potential to be effectively permeated through membranes. It is worth noting, that the HLB value of the prodrug will be determined on the pH of the target's physiological environment. In the stomach where the pH is 1-2, it is expected that amoxicillin **ProD 1-2** and cephalexin **ProD 1-2** will be in the free carboxylic acid forms (a relatively high hydrophobicity) whereas in the blood stream circulation where the pH is 7.4

the carboxylate anion form (a relatively high hydrophilicity) will be the predominant. Our approach was to synthesize amoxicillin **ProD 1-2** and cephalexin **ProD 1-2** as sodium or potassium salts due to their high stability in neutral aqueous medium. It should be emphasized that **1-9** undergo a relatively fast hydrolysis in acidic media whereas they are quite stable at neutral pH.

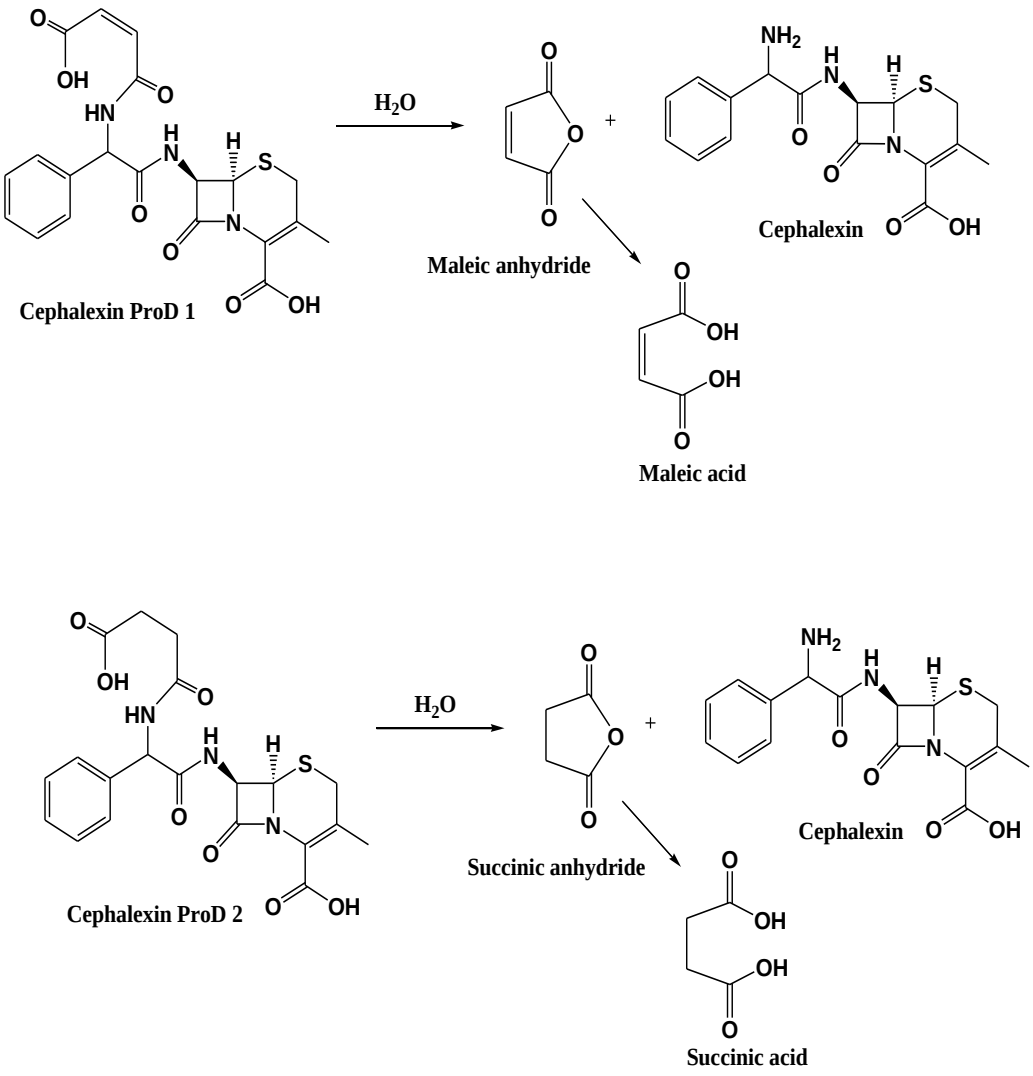


Figure 5: Intraconversion of cephalexin ProD 1-2 to their parent drug, cephalexin.

The designed four prodrugs, amoxicillin **ProD 1-2** and cephalexin **ProD 1-2** were successfully synthesized, according to the schemes shown in Figures 4 and 5, and fully characterized (see experimental section).

The release of the antibacterial agents, amoxicillin and cephalexin, from their corresponding prodrugs, amoxicillin **ProD 1-2** and cephalexin **ProD 1-2** were carried out in aqueous buffers using the same procedure of Kirby *et al.* on N-alkylmaleamic acids **1-9** and the kinetics were

followed by HPLC. This is to investigate whether the antibacterial prodrugs undergo hydrolysis in aqueous medium and to what extent, suggesting the fate of the prodrugs in the system. The kinetics for amoxicillin **ProD 1-2** and cephalexin **ProD 1-2** were carried out in four different aqueous media: 1 N HCl, buffer pH 2.5, buffer pH 5 and buffer pH 7.4. Under the experimental conditions the four antibacterial prodrugs intraconverted to release the parent drugs (Figures 4-5) as was determined by HPLC analysis. For amoxicillin and cephalexin prodrugs, at constant temperature (37℃) and pH the hydrolysis reaction displayed strict first order kinetics as the k_{obs} was quite constant and a straight line was obtained on plotting log concentration of residual prodrug verves time. k_{obs} and the corresponding $t_{1/2}$ for amoxicillin **ProD 1-2** and cephalexin **ProD 1-2** in the different media were calculated from the linear regression equation obtained from the correlation of log concentration of the residual prodrug verses time. The kinetic data for amoxicillin **ProD 1-2** and cephalexin **ProD 1-2** are listed in Tables 1-2. It is worth noting that 1N HCl and pH 2.5 were selected to examine the intraconversion of amoxicillin **ProD 1-2** and cephalexin **ProD 1-2** in the pH as of stomach, since the mean fasting stomach pH of adult is approximately 1-3. Furthermore, environment of buffer pH 5 mimics that of beginning small intestine route, whereas pH 7.4 was selected to determine the intraconversion of the tested prodrugs in the blood circulation system. Acid-catalyzed hydrolysis of amoxicillin **ProD 1-2** and cephalexin **ProD 1-2** was found to be much higher in 1N HCl than at pH 2.5 and 5 (Figures 6-9). At 1N HCl the $t_{1/2}$ values for the intraconversion of amoxicillin **ProD 1** and cephalexin **ProD 1** were about 2.5 hours and that of amoxicillin **ProD 2** and cephalexin **ProD 2** were about 7 and 6 hours, respectively. On the other hand, at pH 7.4, both prodrugs amoxicillin **ProD 1-2** and cephalexin **ProD 1-2** were quite stable and no release of the parent drugs was observed. At pH 5 the hydrolysis of prodrugs amoxicillin **ProD 1-2** and cephalexin **ProD 1-2** was too slow**.** This is because the pK_a of amoxicillin **ProD 1-2** and cephalexin **ProD 1-2** is in the range of 3-4 and it is expected that at pH 5 the anionic form of the prodrug will be dominant and the percentage of the free acidic form that undergoes an acid-catalyzed hydrolysis will be relatively low. In 1N HCl and pH 2.5 most of the prodrug will exist as the free acid form and while at pH 7.4 most of the prodrug will be in the anionic form. The discrepancy in rates between amoxicillin **ProD 1** and amoxicillin **ProD 2** at the different pH buffers is attributed to the strain effects imposed in the case of amoxicillin **ProD 1**, which upon cleavage provides maleic anhydride while in the case of amoxicillin **ProD 2**, the byproduct is the less-strained succinic anhydride. The same picture is also applied for the discrepancy between cephalexin **ProD 1** and cephalexin **ProD 2**. It is worth noting that previous DFT calculations [48] and

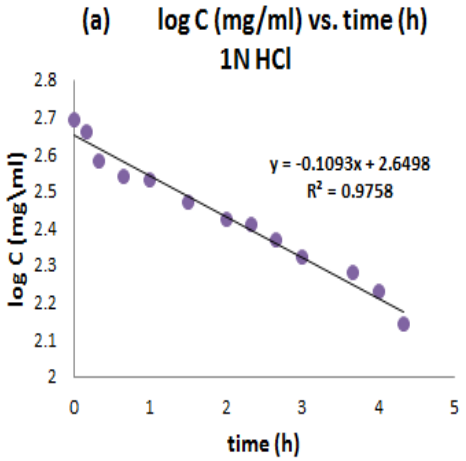
experimental data [41] on the acid-catalyzed hydrolysis of **1-9** revealed that the efficiency of the intramolecular acid-catalyzed hydrolysis by the carboxyl group is remarkably sensitive to the pattern of substitution on the carbon–carbon double bond. The rate of hydrolysis was found to be linearly correlated with the strain energy of the tetrahedral intermediate or the product. Systems having strained tetrahedral intermediates or products experience low rates and vice versa.

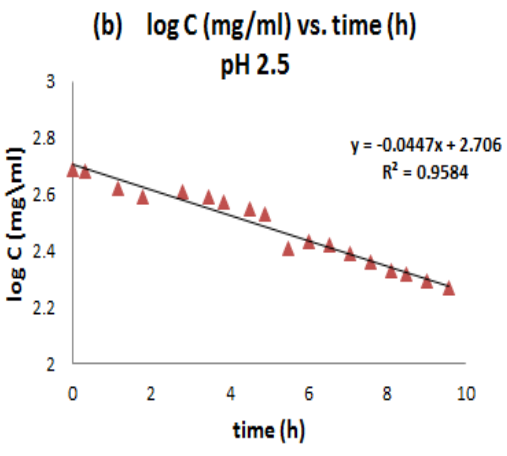
Table 1: Observed *k* and $t_{1/2}$ values for the intraconversion of amoxicillin ProD 1-ProD 2 in 1N HCl and buffers pH 2.5, 5.0 and 7.4.

Medium	k_{obs} (h^{-1})	$t_{1/2}$ (h)	k_{obs} (h^{-1})	$t_{1/2}$ (h)
	Amoxicillin ProD 1	Amoxicillin ProD 1	Amoxicillin ProD 2	Amoxicillin ProD 2
1 N HCl	2.33×10^{-4}	2.5	8.37×10^{-5}	8.2
Buffer pH 2.5	9.60×10^{-5}	7	1.54×10^{-5}	44
Buffer pH 5.0	7.55×10^{-6}	81	No reaction	----
Buffer pH 7.4	No reaction	----	No reaction	----

Table 2: Observed *k* and $t_{1/2}$ values for the intraconversion of cephalexin ProD 1-ProD 2 in 1N HCl and buffers pH 2.5, 5.0 and 7.4.

Medium	k_{obs} (h^{-1})	$t_{1/2}$ (h)	k_{obs} (h^{-1})	$t_{1/2}$ (h)
	Cephalexin ProD 1	Cephalexin ProD 1	Cephalexin ProD 2	Cephalexin ProD 2
1 N HCl	2.41×10^{-4}	2.4	11.38×10^{-5}	6
Buffer pH 2.5	4.17×10^{-5}	14	No reaction	---
Buffer pH 5.0	No reaction	----	No reaction	---
Buffer pH 7.4	No reaction	----	No reaction	----





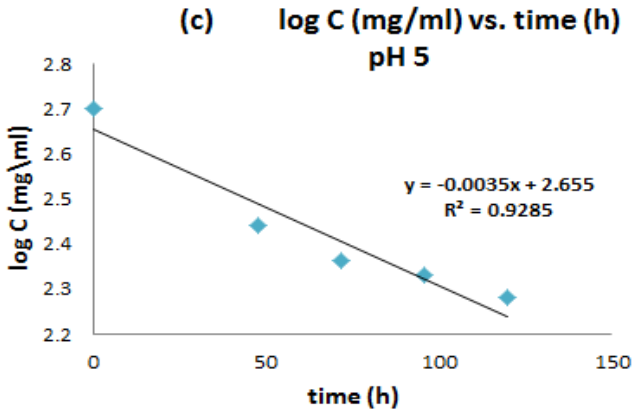


Figure 6: First order hydrolysis plot of amoxicillin ProD 1 in (a) 1N HCl, (b) buffer pH 2.5 and (c) buffer pH 5.0.

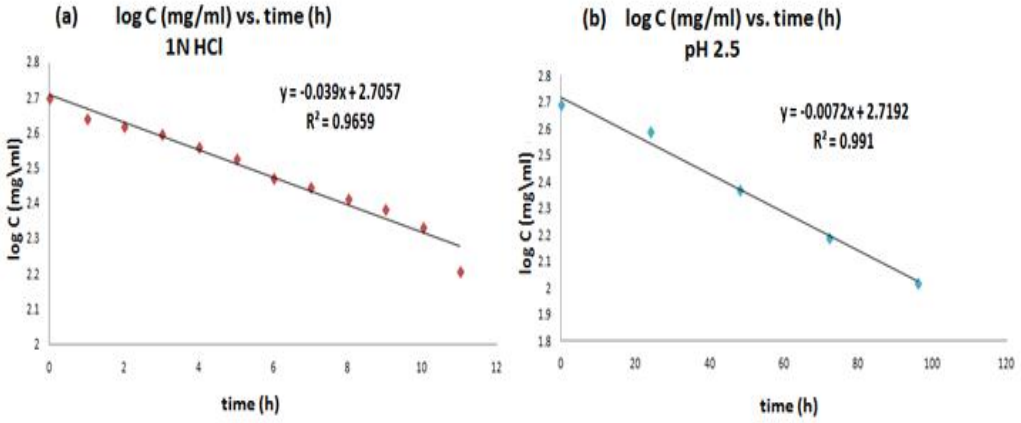


Figure 7: First order hydrolysis plot of amoxicillin ProD 2 in (a) 1N HCl and (b) buffer pH 2.5.

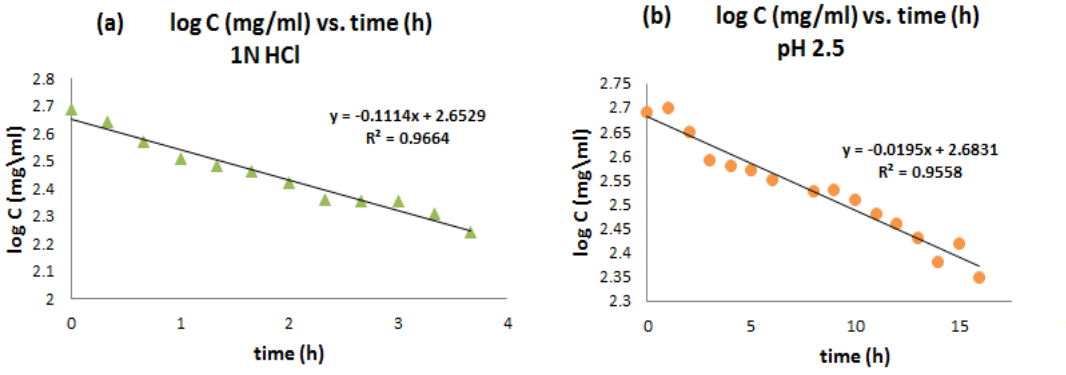


Figure 8: First order hydrolysis plot of cephalexin ProD 1 in (a) 1N HCl and (b) buffer pH 2.5.

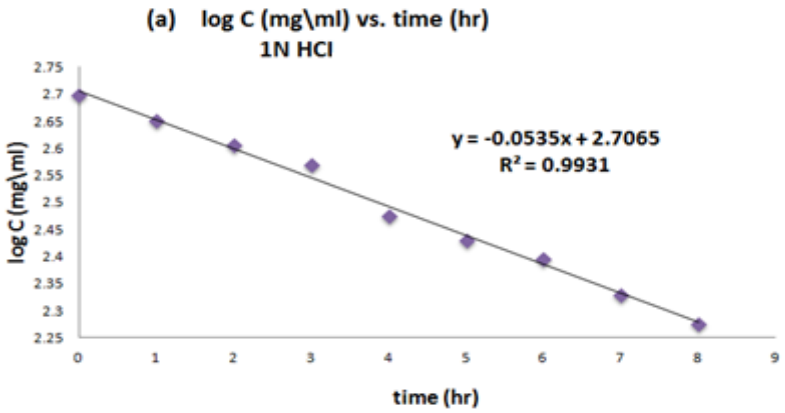


Figure 9: First order hydrolysis plot of cephalexin ProD 2 in 1N HCl.

CONCLUSIONS AND FUTURE DIRECTIONS

The future of prodrug design is forthcoming yet extremely challenging. Progresses must be made in better understanding the chemistry of many organic mechanisms that can be effectively exploited to push forward the development and advances of even more types of prodrugs. The understanding of the organic reactions mechanisms of intramolecular processes will be the next major milestone in this field. It is envisioned that the future of prodrug design holds the ability to produce safe and efficacious delivery of a wide range of active small molecule and biotherapeutics.

Based on Kirby's enzyme model, we utilized two linkers for making novel prodrugs of amoxicillin and cephalexin, with the expectation to have prodrugs lacking the bitter sensation of their parent drugs as well as to be cleaved in different rates. The quantum mechanics (QM) calculations using different methods revealed that the acid-catalyzed hydrolysis efficiency of processes **1-9**, amoxicillin **ProD 1-2** and cephalexin **ProD 1-2** is significantly sensitive to the pattern of substitution on the carbon-carbon double bond and nature of the amine leaving group. According to DFT calculations, the four antibacterial prodrugs will exist as a free carboxylic acid form (a relatively high lipophilicity) in the stomach, whereas in the blood circulation system, the carboxylate anion form (a relatively low lipophilicity) will be predominant. The synthesized prodrugs, amoxicillin **ProD 1-2** and cephalexin **ProD 1-2,** were found to undergo hydrolysis in acidic aqueous medium, whereas they were stable at pH 7.4. The predicted $t_{1/2}$ and k_{obs} of amoxicillin **ProD 1-2** and cephalexin **ProD 1-2** were calculated. Kinetics studies on the interconversion of the newly synthesized amoxicillin and cephalexin prodrugs revealed that the $t_{1/2}$ was largely affected by the pH medium as predicted.

In vitro binding test has shown that the four antibacterial prodrugs, amoxicillin **ProD1-ProD2** and cephalexin **ProD1-ProD2**, do not bind to bitter taste receptors. It is believed that the addition of the linker (maleic or succinic promoiety) to amoxicillin and cephalexin hinders the ability and capability of the prodrug to interact with the active sites of the bitter taste receptors. In addition, in vitro antibacterial testing of amoxicillin ProD 2 and cephalexin ProD 2 demonstrated that both prodrugs possess strong antibacterial activity against a variety of bacteria.[117]

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