Synthesis and Preliminary Kinetic Study of 5-fluorouracil Derivatives for Targeting Colon Tumor

Mohammed H. Mohammed [1], Firas Aziz Rahi [2], Hasanain Amer Naji [3]

[1]Department of Pharmaceutical Chemistry, Collage of pharmacy, University of Baghdad, Baghdad, Iraq.
[2]Department of Pharmaceutics, Collage of Pharmacy, University of Al-Mustansiriyah, Baghdad, Iraq.
[3]Department of Pharmaceutical Chemistry, Collage of Pharmacy, University of Al-Mustansiriyah, Baghdad, Iraq.

*Author to whom correspondence should be addressed; E-Mail: hasanainaltaweel@gmail.com

Abstract

5-Fluorouracil (5-FU) is used widely as an anticancer drug to treat solid cancers, such as colon, breast, rectal, and pancreatic cancers; although it's clinical application is greatly limited by its short plasma half-life, poor tumor affinity, myelosuppression, and gastrointestinal toxicity. To tackle these problems, numerous modifications of the 5- Fu structure have been performed.Thus, 5-Fu as possible colon specific prodrugs were synthesized in which 5-Fu is attached to amino acids (alanine & phenylalanine) using succinate group as a spacer via amide or ester bond. An approach to improve the properties of 5-fluorouracil is the chemical transformation into bioreversible derivatives (prodrugs) which are converted to the parent drug by enzymatic and / or chemical hydrolysis. The synthesis of the target compounds were accomplished following multistep reaction procedures, the chemical reaction followed up and the purity of the products were checked by TLC.

The structures of the final compounds were confirmed by their melting points, infrared spectroscopy and ^{1}H-NMR spectra. The hydrolysis of compounds III, IV, V, and VI in aqueous buffer solutions of pH 1.2 and pH 7.4 were studied.

Compounds III, IV, V and VI had enough stability at pH 1.2 (t = 429.874min, t =429.874min, t=334.336min and t =376.139min respectively) and at pH 7.4 (t=601.823min, t=601.823min, t=429.874min and t=501.519min respectively); expecting that hydrolysis of these compounds by microbial enzymes in the colon will deliver 5-fluorouracil spontaneously.

Indexing terms/Keywords

5-fluorouracil; colon specific prodrug ; amino acids; kinetic study.

Academic Discipline And Sub-Disciplines

Pharmaceutical chemistry

SUBJECT CLASSIFICATION

Heterocyclic compounds

TYPE (METHOD/APPROACH)

Synthesis, characterization and kinetic study

Introduction

Colon cancer is the second cause of cancer related deaths in the world. Although improvements have been made in surgical techniques and in chemotherapies, the survival rate is still low [1]. 5-fluorouracil is an antimetabolite of the pyrimidine analogue type, which is frequently used for treating solid tumors, such as colorectal, gastric tract, and liver carcinomas [2, 3].

However, the clinical applications of 5-FU are greatly limited by its short plasma half-life, poor tumor affinity, myelosuppression, and strong intestinal toxicity. To tackle these problems, numerous modifications of the 5- Fu structure have been performed, thus, a series of 5-Fu prodrugs in which 5-Fu is attached to amino acids, peptides, phospholipids, and polymers have been reported [4-7].

The term "prodrug", first introduced by Albert, refers to a chemically modified form of a drug [8] that is devoid of pharmacological activity, but that can be converted to the active form of the drug in a biological system, where it exerts the desired action. This strategy can improve the limitations associated with the effective transport into tumor cells, catabolic inactivation before the cytotoxic entity can reach the tumor, and short plasma half-1ife [9, 10].

As the site of drug absorption, the colon holds several drawbacks, such as small surface area, low fluidity of the lipid membrane, existence of tighter junction compared to the small intestine, low motility, and high viscosity of intestinal contents. On the other hand, long transit time, low proteolytic activity, and responsiveness to the action of absorption enhancers are the positive aspects of the large intestine as the site of drug absorption compared to the small intestine [11].

The objective of the colon-targeted drug delivery is to avoid absorption and degradation of drugs in the upper GI tract allowing them act site-specifically in the colon and this achieved by various approaches such as pH dependent utilizing the changes in pH along the GI tract [12], coated dosage forms [13], time-controlled release systems [14], pressure-controlled colon delivery systems [15], coating drugs with bacterially degradable polymer [16] and prodrug based drug delivery system [17]. The colonic drug delivery systems are mainly based on degradation of a prodrug by microorganisms of the colon flora [18] and colonic enzymes [19].

To design a colon-specific prodrug, a parent drug is usually linked to a nonabsorbable carrier such as a polymeric matrix or a hydrophilic small molecule to prevent the absorption of the colon-specific prodrug in the upper intestine [20].

Ho et al. [21] reported that the liver has the highest dihydrouracil dehydrogenase activity with minimal activity in the colon and colon tumor. These studies suggest that bioactivation of 5-FU to 5-fluoro-2-deoxyuridine is most active and bioinactivation of 5-FU to dihydro-5-FU is the least active in colon tumor. If 5-FU is delivered specifically to colon, the active metabolite of 5-FU should be most available in colon tumor, and the systemic side effects of 5-FU will be minimized compared with oral administration.

Spacer or linkers can be used if the desired prodrug promoiety cannot be attached directly to the parent molecule due to a sterical hindrance or functional properties of the parent drug. The spacers increase the distance between the parent molecule and the promoiety and are usually cleaved spontaneously after the enzymatic or chemical decomposition of the prodrug bond between the promoiety and the spacer [22].

EXPERIMENTAL PROCEDURE

Synthesis of methyl ester hydrochlorides of L-phenylalanine and L-Alanine:

A suspension of L-Phenylalanine (9 mmole) dissolved in (15 mL) of absolute methanol, was cooled down to -15 ^{0}C then thionyl chloride was added drop wise (9 mmole), (the temperature should be keep below -10^{0}C), the reaction mixture was left at 40^{0}C for 1hr, then refluxed for 4hr and left at room temperature overnight, , It was titrated with 20 ml portion of cold ether at 0 ^{0}C until the excess of dimethyl sulphate was removed, the excess solvent was evaporated to dryness under vacuum, re-crystallize the product from methanol-petroleum ether to get pure L-Phenylalanine methyl ester HCl. The same procedure was followed to synthesize L-Alanine methyl ester HCl [23].

(S)-methyl-2-amino-3-phenylpropanoate hydrochloride (A).

As White crystal (87%yield); m.p.160 c°. $R_f$value=0.65. IR 3425-2623of (NH$_3^+$Cl$^-$), 1747(C=O) of ester, 1629asym. bending of (NH$_3$), 1242 and 1145 (C-O-C) cm^{-1} , 742 and 702 (C-H) out of plane bending vibration of monosubstituted benzene.

(S)-methyl 2-amino propanoate hydrochloride (B).

As White crystal (82%yield); m.p. 120 c°. $R_f$value=0.7. IR 3439-2700 (NH$_3^+$Cl$^-$), 1741 (C=O) of ester, 1618 bending of (NH$_3$), 1506 (C=C) aromatic, 1253 and 1199 (C-O-C) cm^{-1}.

Synthesis of Intermediate (I):

L-Phenylalanine methyl ester HCl (2mmole) was treated with (2mmole) of succinic anhydride in tetrahydrofuran and (2mmole) of triethylamine at room temperature for 10 min. The precipitate was filtered and dried without further purification to get Intermediate (IA). The same procedure was followed with L-Alanine methyl ester HCl to synthesize Intermediate (IB) [24].

(S)-4-(1-methoxy-1-oxo-3-phenylpropan-2-yl amino)-4-oxobutanoic acid Intermediate (IA)

As white powder (75%yield); m.p.210-212°C decompose.R$_f$value=0.75, IR(KBr cm^{-1}) 3440 NH of 2^0 amide, 2500-3000 OH str. vibr., 2976, 2941(C-H) of CH3 and CH2, 1745 broad (C=O)of ester and acid, 1653 (C=O) of 2^0 amide, 1539 (N-H) bending of 2^0 amide, 1477 (C=C) of benzene, 1442&1394 (C-H) bend of CH2 & CH3, 1172 (C-O), 1240 asym.(C–O–C), 750 &702 (C-H) out of plane bending vibration of monosubstituted benzene.

(S)-4-(1-methoxy-1-oxopropan-2-yl amino)-4-oxobutanoic acid Intermediate (IB)

As white powder (70%yield); m.p.198-200°C decompose.R$_f$value=0.79, IR(KBr cm^{-1}) 3381 NH of 2^0 amide, 2500-3000 OH str. vibr. , 2978, 2941 (C-H) of CH3& CH2, 1734 (C=O) stretching vibration of ester and acid, 1622 (C=O) str. of 2^0 amide, 1593 (N-H) bending of 2^0 amide, 1448, 1400&1363 (C-H) bend of CH3 & CH2, 1170(C-O), 1232 asym.(C–O–C).

Synthesis of Intermediate (II):

A suspension of intermediate (IA) (2mmole, 0.56g) dissolved in (10ml) of dry chloroform, was cooled down to -15^{0}C then thionyl chloride (2mmole, 0.15mL) was added slowly to it. The mixture was refluxed for 4hr at 60-70^{0}C with continuous stirring. Then evaporate the excess solvent by using rotary evaporator. The viscous liquid was slowly added drop wise into a beaker contain of 2,4–dinitrophenol(2mmole, 0.368g) dissolved in (5ml) of dry chloroform and triethylamine (2mmole, 0.28ml) for 1hr on an ice bath, then continuous stirring overnight at room temperature. The solid that separated out was filtered off and washed with dry chloroform to give Intermediate (IIC). The same procedure was followed with Intermediate (IB) to give Intermediate (IID) [25].

(S)-2,4-dinitrophenyl4-(1-methoxy-1-oxo-3-phenylpropan-2-yl amino)-4-oxobutanoate. Intermediate (IIC)

As yellowish powder (62%yield);m.p.77-78°C.R$_f$value=0.64, IR(KBr cm^{-1}) 3269 (N-H) of 2^0 amide, 3109 (C-H) of aromatic, 2978, 2945 (C-H) of CH3&CH2, 1743 & 1710 (C=O) of ester, 1626 (C=O) of 2^0 amide, 1597(N-H) bending of 2^0 amide , 1479 (C=C) of aromatic, 1537 (NO2)anti symm., 1340 (NO2)symm., 1178 (C-O) , 1255 asym. (C–O–C), 1437&1398 (C-H) bend of CH3&CH2.

(S)-2,4-dinitrophenyl4-(1-methoxy-1-oxopropan-2-ylamino)-4-oxobutanoate. Intermediate (IID)

As brownish powder (65%yield);m.p. 68-70°C.R$_f$value=0.57, IR(KBr cm^{-1}) 3269 (N-H) of 2^0 amide, 3107 (C-H) of aromatic, 2976, 2939 (C-H) of CH3&CH2, 1739 (C=O) stretching vibration of ester, 1626 (C=O) of 2^0 amide, 1599 (N-H) bending of 2^0 amide, 1477 (C=C) of aromatic, 1537 (NO2) anti symm. , 1340 (NO2) symm., 1180 (C-O), 1257 asymm. (C–O–C), 1435&1394 (C-H) bend of CH3&CH2.

Synthesis of compound (III) & (IV):

A suspension of intermediate (IA) (2mmole, 0.56g) dissolved in (10ml) of dry chloroform, and was cooled down to -15^{0}C then thionyl chloride (2mmole, 0.15mL) was added slowly to it. The mixture was refluxed for 4hr at 60-70^{0}C with continuous stirring. Then evaporate the excess solvent by using rotary evaporator. The viscous liquid was slowly added drop wise into a beaker contain 5-fluorouracil (2mmole, 0.26g) dissolved in a dry 1,4 dioxane (5mL) and triethylamine (2mmole, 0.28ml) for 1hr on an ice bath, then stirring overnight at room temperature. The obtained suspension was filtered and the filtrate was washed with distilled water (20ml), dried with anhydrous magnesium sulfate and the solvent was evaporated by vacuum. The obtained compound was crystallized from diethyl ether-petroleum ether to give compound (III). The same procedure was followed with Intermediate (IB) to give compound (IV) [26].

Methyl2-(4-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-oxobutanamido)-3-phenylpropanoate. Compound (III)

As white powder (77%yield);m.p. 175-176°C.R$_f$value=0.82, IR (KBr cm^{-1}) 3263 (N-H) of 2^{0}amide, 3142 (N-H) of pyrimidine, 3059 (C-H) of aromatic , 2987 & 2827 (C-H)of CH3&CH2, 1718, 1479 & 1394 NH(C=O)-C=C-amide I, II and III (str. vibr.) of uracil and C=C of benzene ring,1668 (C=O) of 2^0 amide,1247 (C-F)1213 asym.(C–O–C), 1440 (C-H) bend of CH3 & CH2, 1170 (C-O), 750 and 715 (C-H) out of plane bending vibration of monosubstituted benzene. ^{1}H-NMR (300MZ, DMSO-d$_6$):11.8 (1H, NH* of 5-FU), 10.3 (1H, NH* of CONH), 8.413 (1H, CH* of FC=CH), 7.587 - 6.135 (5H, Ar-H*), 4.558 (1H, CH* of CHNHCO), 3.165 (3H, OCH$^*_3$), 2.888-2.867 (2H, CH$^*_2$-Ar), 1.009-0.987 (4H, CH$^*_2$CH$^*_2$).

Methyl2-(4-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-oxobutanamido)-3-propanoate. Compound (IV)

As white powder (80%yield);m.p. 164-166°C.R$_f$value=0.8, IR (KBr cm^{-1}) 3136 (N-H) of 2^0 amide,3064(N-H)of pyrimidine, 2978, 2937 (C-H) of CH3&CH2, 1718, 1479 & 1394 NH(C=O)-C=C-amide I, II, and III (str.vib.) of uracil, 1668 (C=O of 2^0 amide,1249 (C-F),1213 asym.(C–O–C), 1437 (C-H) bend of CH3 & CH2,1172 (C-O). . ^{1}H-NMR (300MZ, DMSO-d$_6$): 11.5 (1H, NH* of 5-FU), 10.6 (1H, NH* of CONH), 7.75 (1H, CH* of FC=CH), 3.68 (1H, CH* of CHNHCO), 3.17 (3H, OCH$^*_3$), 1.37-1.18 (7H, CH$^*_2$ CH$^*_2$, CCH$^*_3$).

Synthesis of Compound (V) & (VI):

The reaction of Intermediate (IIC) (1 mmole, 0.446g) and 5-fluorouracil as sodium salt (2mmole, 0.306g) in absolute ethanol (10ml) was refluxed for three hours at 65^{0}C. The formed precipitate was filtered, washed with cold ethanol and re-crystallized from ethanol as a pale yellow crystal of compound (V). The same procedure was followed with Intermediate (IID) to give pale brown crystals of compound (VI) [27].

5-fluoro-6-oxo-1,6-dihydropyrimidine-2-yl4(1-methoxy-1-oxo-3-phenylpropan-2-ylamino)-4-oxobutanoate. Compound (V)

As pale yellow powder (60%yield);m.p.129-130°C.R$_f$value=0.7, IR (KBr cm^{-1}) 3128 (N-H) of 2^0 amide,3068(N-H) of pyrimidine, 2982, 2937& 2825 (C-H) of CH3&CH2, 1724, 1483 & 1396 NH(C=O)-C=C-amide I, II, and III (str.vib.) of uracil and C=C of benzene ring, 1664 (C=O) of 2^0 amide, 1247 (C-F), 1176 asymm. (C–O–C), 1435 & 1336(C-H) bend of CH3 & CH2, 1128(C-O), 752 and 719 (C-H) out of plane bending vibration of monosubstituted benzene. ^{1}H-NMR (300MZ, DMSO-d$_6$): 11.30 (1H, NH* of 5-FU), 10.4 (1H, NH* of CONH), 8.416 (1H, CH* of FC=CH), 7.584 - 6.118 (5H, Ar-H*), 4.286 (1H, CH* of CHNHCO), 3.162 (3H, OCH$^*_3$), 2.851-2.827 (2H, CH$^*_2$-A r), 1.018-0.972 (4H, CH$^*_2$CH$^*_2$).

### 5-fluoro-6-oxo-1,6-dihydropyrimidine-2-yl4(1-methoxy-1-oxopropan-2-ylamino)-4-oxobutanoate. Compound (VI)

As pale brown powder (55%yield); m.p.140-142°C.R$_f$value=0.77, IR (KBr cm^{-1}) 3132 (N-H) of 2^0 amide,3066(N-H) of pyrimidine, 2980,2937(C-H) of CH3&CH2, 1724, 1483& 1396 NH(C=O)-C=C-amide I, II, and III (str.vib.) of uracil,1662 (C=O) of 2^0 amide,1247 (C-F),1176 asymm. (C–O–C), 1435 & 1325(C-H) bend of CH3 & CH2, 1078(C-O). ^{1}H-NMR (300MZ, DMSO-d$_6$): 11.50 (1H, NH* of 5-FU), 10.6 (1H, NH* of CONH), 7.75 (1H, CH* of FC=CH), 3.32 (1H, CH* of CHNHCO), 3.08 (3H, OCH$^*_3$), 1.22-0.99 (7H, CH$^*_2$CH$^*_2$, CCH$^*_3$).

Preliminary kinetic study of 5-fluorouracil derivatives at pH 1.2, pH 7.4:

The stability of 5-fluorouracil derivatives was studied in (0.2M) HCl solution (pH 1.2) [85ml (0.2M) HCl + 50ml (0.2 M) KCl, Distilled water, ad. to 200ml] and in (0.1M) phosphate buffer solution (pH 7.4) [100ml (0.1 M) KH2PO4 + 78.2ml (0.1 M) NaOH, Distilled water, ad. to 200ml] incubated at 37°C. The total buffer concentration was 0.1 M and the ionic strength (μ) 1.0 was maintained for each buffer by addition of calculated amount of NaCl. The rate of hydrolysis was followed spectrophotometrically (UV method) by recording the decreases in the absorbance of 5-fluorouracil derivatives accompanying the hydrolysis. The reactions were initiated by adding 1 ml of stock solutions (1mg /ml) of the derivatives in methanol to preheated buffer solution at 37°C to give final concentration of derivatives (0.02mg /ml). The solutions were kept in a water bath at 37°C and samples (3ml) were withdrawn at appropriate time interval (15, 30, 60,120 and 240 min.) and the absorbances were recorded. The observed first rate constants were determined from the slopes of the linear plots of log. concentration remaining versus time [28].

Results and Discussion

The synthetic pathways for the designed target compounds (III- VI) are illustrated in (scheme 1)

Scheme1. Synthesis of target compounds (III- VI) & their intermediates.

Intermediate (IA, IIC) (If R=   Phenylalanine)
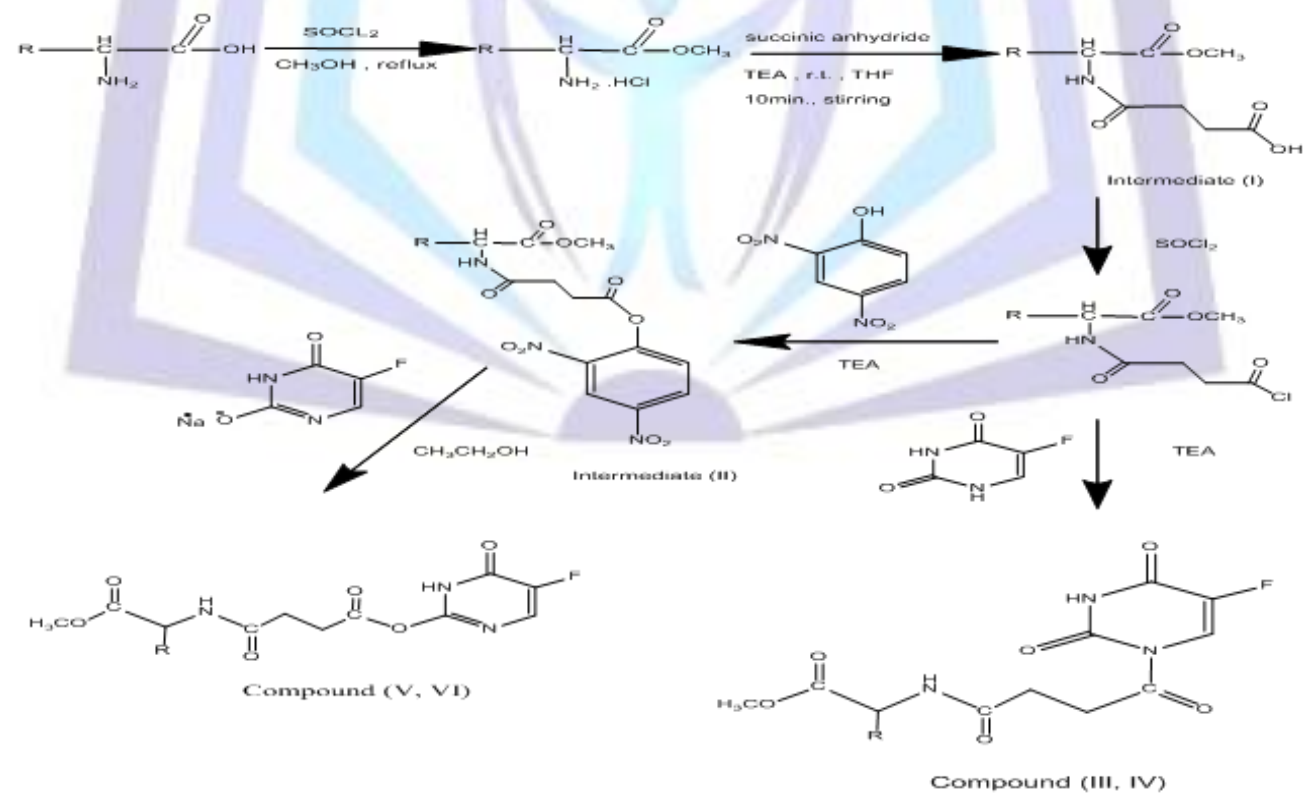
Compound (III, V)

Intermediate (**IB, IID**)   (if   R=CH$_3$ Alanine)

Compound (**IV**, **VI**)

Kinetic study:

The λmax of fluorouracil 265 nm was the same λmax of compounds **III, IV, V** and **VI,** but they differ in molar absorpitivity (**E**) according to the Beer-Lambert equation [29]:

A=**E** c b -------- equation (1)

Thus making UV method relevant for studying the hydrolysis of these analogues, so the amount of fluorouracil alone was calculated from the following equation [29]:

A$_T$ = **E**$_R$ [R]-**E**$_R$ [P] + **E**$_P$ [P] -------- equation (2)

Where:

A$_T$= absorption at any time

E_R= the molar absorpitivity of compound III, IV, V and VI at pH 1.2 & 7.4

[R]= the initial concentration of each compound III, IV, V and VI

E_P= the molar absorpitivity of 5-FU at pH 1.2 & 7.4

[P]= the concentration of 5-FU

Under experimental conditions used the hydrolysis of the 5-fluorouracil derivatives followed pseudo first order kinetics, since plots of log concentration of 5-fluorouracil alone vs. time resulted in straight lines, from their slopes; the observed rate constants of hydrolysis were calculated. Figures (1), (2) ,(3) and (4) are represented graphs for pH stability profile of the compounds **III, IV**, **V** and **VI** respectively; while table (1) shows the pH values, the corresponding K$_{obs}$ and half-life of the hydrolysis of 5-fluorouracil analogues. The half-life was calculated using equation (4), which derives from the first order kinetic law [equation (3)].

log. C = log. C$_o$ – k t / 2.303 -------- equation (3)

t $_{1/2}$ = 0.693 / Kobs   -------- equation (4)

Table (1) the rate constant of hydrolysis of compounds III, IV, V and VI at pH 1.2 and pH 7.4 at 37°C.

compound	pH	K$_{obs}$(min^{-1})	t½(min)
III	1.2 7.4	1.6121×10^{-3} 1.1515×10^{-3}	429.874 601.823
IV	1.2 7.4	1.6121×10^{-3} 1.1515×10^{-3}	429.874 601.823
V	1.2 7.4	2.0727×10^{-3} 1.6121×10^{-3}	334.346 429.874
VI	1.2 7.4	1.8424×10^{-3} 1.3818×10^{-3}	376.139 501.519

One of the crucial requirements for a prodrug is that used, it should show a good stability in aqueous solutions and in gastrointestinal fluid, and it should be readily hydrolyzed following gastrointestinal absorption to release the parent drug [30].
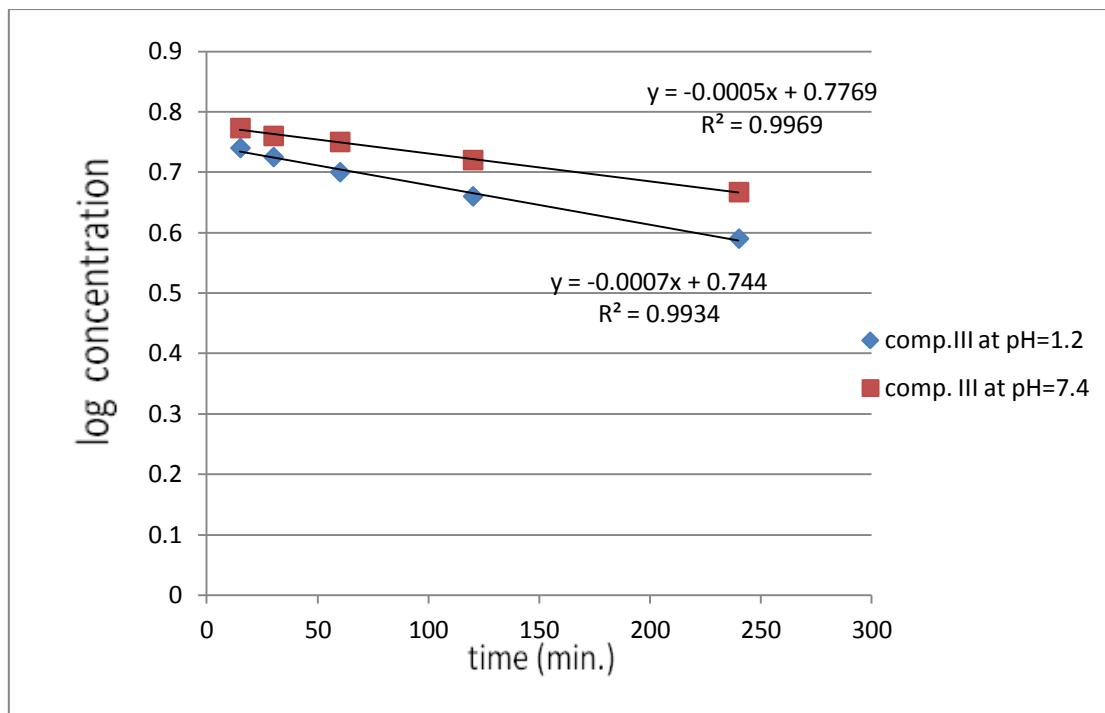
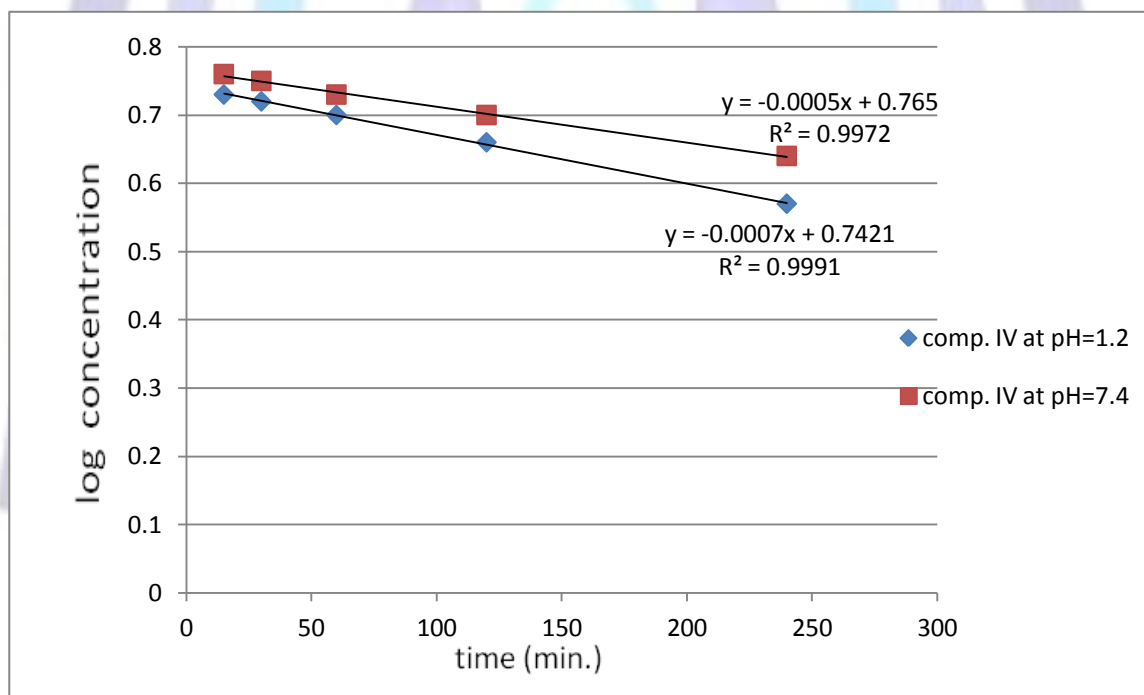**Figure (1) the hydrolysis of compound III in 0.1M HCl solution (pH 1.2) and phosphate buffer (pH 7.4) at 37°C**

Figure (2) the hydrolysis of compound IV in 0.1M HCl solution (pH 1.2) and phosphate buffer (pH 7.4) at 37°C
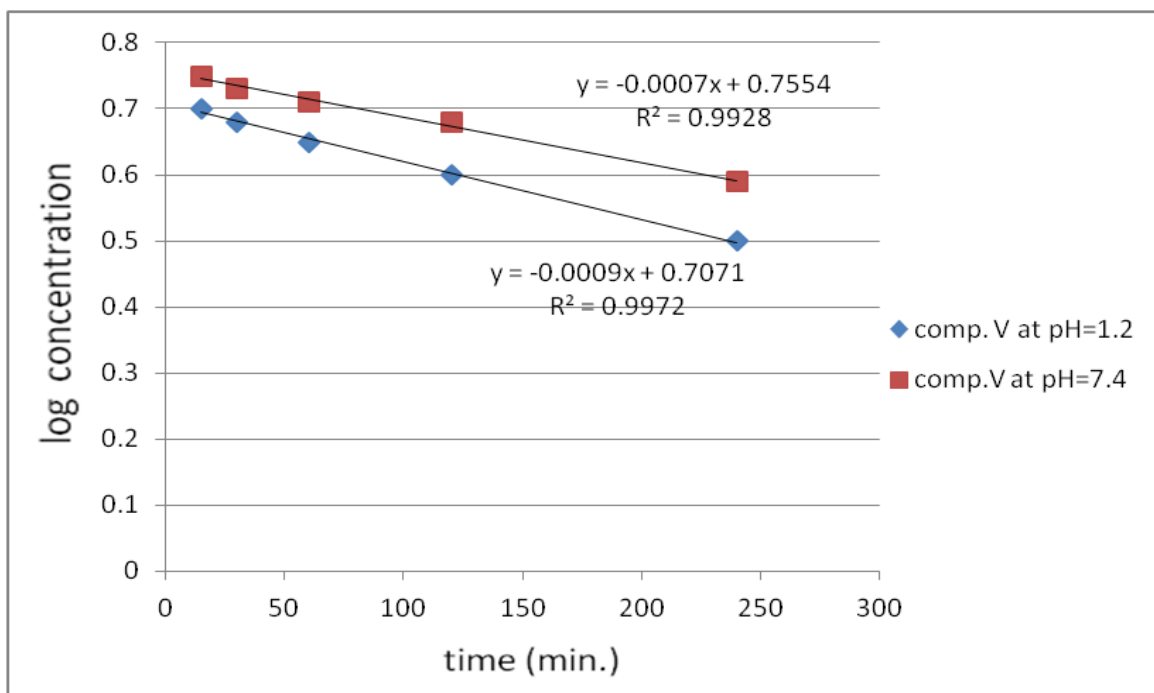
$$y = -0.0007x + 0.7554$$
$$R^2 = 0.9928$$

$$y = -0.0009x + 0.7071$$
$$R^2 = 0.9972$$

Figure (3) the hydrolysis of compound V in 0.1M HCl solution (pH 1.2) and phosphate buffer (pH 7.4) at 37°C

$$y = -0.0006x + 0.76$$
$$R^2 = 0.9972$$

$$y = -0.0008x + 0.7142$$
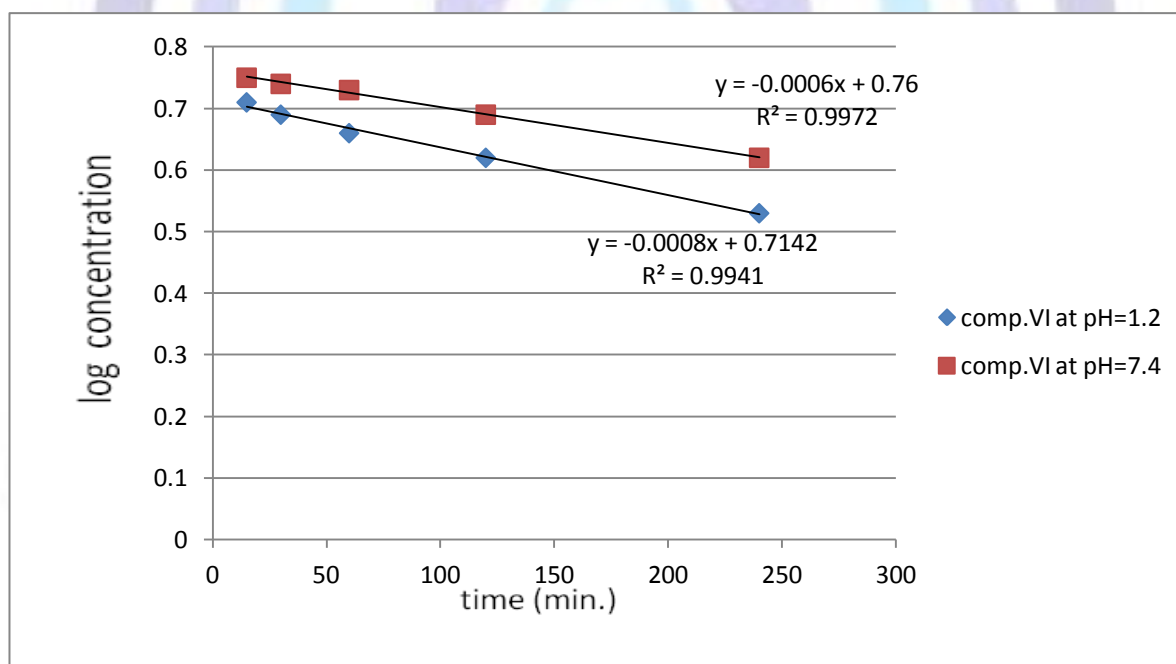$$R^2 = 0.9941$$

Figure (4) the hydrolysis of compound VI in 0.1M HCl solution (pH 1.2) and phosphate buffer (pH 7.4) at 37°C

Conclusion:

The synthetic procedure for the designed target compound was successfully achieved and the structural formula for the synthetic compound was characterized using IR spectroscopy, ^{1}H-NMR, melting points and R_f values. Preliminary kinetic study for compounds **III, IV, V** and **VI** revealed that these compounds were stable at pH 1.2 and pH 7.4.

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