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Synthesis of Novel Ester-Based 5-Fluorouracil Derivatives

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Abstract

Fluorouracil (5-Fu, **1**) is an antimetabolite cancer drug and the first-line drug of anticancer administration by WHO that has been widely used worldwide for more than 50 years. The development of 5-fluorouracil is an effort to obtain higher activity, decrease side effects, and create a specific target receptor compared to 5-fluorouracil. In this research, a series of novel 5-Fluorouracil (5-Fu) derivatives has been synthesized based on a benzoylation reaction (Schotten-Baumann reaction) of N1-hydroxylated 5-Fu called compound **2**, 5-fluoro-1-(hydroxymethyl)-uracil. The benzoyl chloride substituents used in this research, including 3-chlorobenzoyl (**4a**), 2-chlorobenzoyl (**4b**), 3-nitrobenzoyl (**4c**), 4-methoxy benzoyl (**4d**), 4-trifluoromethylbenzoyl (**4e**), 3,4-dichlorobenzoyl (**4f**), and 4-nitrobenzoyl (**4g**) chloride become (**4a-4f**)-5Fu. This research meticulously examined the conditions (time and reaction temperature) at the second step of the synthesis reaction (esterification), ensuring the reliability of the results. The best synthesis conditions for **4a**, **4b**, **4c**, **4d**, and **4g** compounds were found to be reflux at 40°C for 6 hours, whereas **4e** and **4f** compounds reactions were performed in an ice bath for 11 and 17 hours, respectively. All product syntheses, **4a-4g** compound, were purified using column chromatography and eluted using eluent hexane: acetone (6:4), and the yields of **4a-4g** compounds were around 61-79%. The pure compounds were characterized using FTIR and ^{1}HNMR spectrometer, further validating the research. Based on these findings, it can be concluded that all 5-Fu derivatives can be synthesized using the Schotten-Baumann reaction method.

1. Introduction

5-Fluorouracil (5-Fu, **1**) is an antimetabolite cancer drug and the first-line drug of anticancer administration by WHO that has been widely used worldwide for more than 50 years [1, 2]. The effectiveness of 5-Fu may be supported by the synergistic effect of other drugs to act as co-treatment, resulting in an antitumor effect against carcinoma cells [3]. As a prodrug, 5-Fu has a relatively narrow therapeutic window; hence, it is frequently used as standard chemotherapy for the adjuvant treatment of some solid tumors, such as colorectal, gastric tract, and liver carcinomas. The mechanism of action and metabolism of 5-Fu has been proposed in two routes: anabolic and catabolic. By infusion intake, 5-Fu is converted to active metabolite fluorodeoxyuridine triphosphate (FUTP) or fluorodeoxyuridine diphosphate (FdUDP) to inhibit thymidylate synthase (TS) in the cells. It is also catabolized into dihydrofluorouracil (DHFU) in the liver, which results in smaller metabolites excreted through the kidneys when 5-Fu is consumed as oral intake [2, 4, 5, 6]. Drug development of 5-Fu is an effort to obtain higher activity, decrease side effects, and a specific target receptor compared to 5-Fu. However, 5-Fu shows a short plasma half-life, poor tumor affinity, and wide inter-patient variability in toxicity and severity, like mucositis, nausea, emesis, myelosuppression, diarrhea, neutropenia, leukopenia, and hand-foot syndrome, associated with toxic cardiac reaction. The extensive study of this 5-Fu toxicity has been investigated by many researchers [7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19]. Therefore, the availability of 5-Fu derivatives that have

better pharmacokinetic performance and less toxic effects is required.

Numerous efforts have focused on the synthesizing of 5-Fu derivatives having better properties than the lead compound, 5-Fu. Development of 5-Fu derivatives mainly focused on synthesizing high molecule polymer and macromolecule agents containing 5-Fu and nucleoside derivatives [20]. Some literature has also reported the synthesis of 5-Fu derivatives via substitution of amino acids, peptides, phospholipids, esters, and polymers on the *N* atoms (N1 and/or N3). Most of those compounds show the enhancement of pharmacology and pharmacokinetics properties compared to 5-Fu in terms of bioactivity, selectivity, metabolic stability, and absorptivity, and the reduction of the side effects [4, 12, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30].

One favorable technique for synthesizing 5-Fu derivatives is the esterification of 5-Fu intermediate compounds, such as N1-hydroxymethylene-5-fluorouracil **2** or 5-fluorouracil-1-yl acetic acid, with an organic compound, such as carboxylic acid or amino acid ester [21, 22] or other drugs, such as nitrogen mustard and sulfadiazine [31]. This technique has been studied comprehensively since ester is easy to prepare and shows satisfactory physicochemical parameters, especially for water solubility properties. Instead of enhancing the water solubility, other common works involving esterification of 5-Fu are aimed to improve drug release rate, drug loading, thermo sensitivity, and to prolong the duration of the activity [31, 32, 33]. However, the formation of ester via esterification requires using a catalyst to enhance the kinetics of the reaction. Once the selection of catalyst is unsuitable for the reaction, it may result in the formation of a by-product that is hard to remove from the synthesis product and lower the percentage of yield [22].

Benzoylation (Schotten-Baumann reaction) is a reaction that introduces a benzoyl group into an organic compound and is mostly used to form an ester or amide. This reaction is included in the category of the most important transformations in organic synthesis [34, 35, 36]. Since the benzoyl group is reactive, forming ester based on this reaction is more favorable than based on esterification. In addition, generally, benzoylation does not require a catalyst to run the reaction [37, 38].

In the previous study related to benzoylation, Tian *et al.* [22] and Puspaningtyas [37] synthesized a 5-Fu derivative by substitution of 4-chlorobenzoyl substituent on **1**. This ester-based 5-Fu derivative shows high *in vitro* anticancer activity. Therefore, based on the Topliss method [38, 39], substituent replacements that can be used instead of 4-chlorobenzoyl are 4-nitrobenzoyl, 3,4-dichlorobenzoyl, and 4-trifluorocarbonbenzoyl. However, recent work from the Institute of Tropical Disease (ITD), Airlangga University, suggests that the substitution of 4-chlorobenzoyl on **1** showed low anticancer activity when it was tested by *in vivo* method in mice. Thus, this study also aims to assess a replacement of 4-chlorobenzoyl as a substituent on 5-Fu through the Topliss approach using other substituents, i.e. 2-chloro, 3-chloro, 3-nitro, and 4-methoxy benzoyl.

According to the information mentioned earlier, this research explores the possibility of synthesizing a series of ester-based 5-Fu derivatives via benzoylation of **1** using various benzoyl substituents, and we hope that drug development of 5-Fu becomes ester-based 5- fluorouracil derivatives can obtain higher activity, decrease side effects and a specific target receptor compared to 5-Fu as a lead compound. These compounds are examined for their physical and chemical characteristics using melting point apparatus, TLC-densitometry, FTIR, and ^{1}HNMR.

2. Experimental

2.1. Materials

The materials used include 5-fluorouracil (AR, Sigma), 3,4-dichlorobenzoyl chloride (Sigma), 4-trifluoromethylbenzoyl chloride (Sigma), 3-nitrobenzoil chloride (Sigma), 4-methoxybenzoyl chloride (Sigma), 3-chlorobenzoyl chloride (Sigma), 2-chlorobenzoyl chloride (Sigma), formaldehyde (pro synthesis), triethylamine (Merck), acetone (Merck), ether (Merck), chloroform (Merck), silica gel 60 F 254, diethyl ether (Merck), chloroform (Merck), methanol (Merck), ethyl acetate (Merck), acetone (Merck), methanol (Merck), distilled water, KBr pro spectrometry, D_3CCOCD_3 (acetone-d_6) pro NMR, and tetramethylsilane pro NMR.

2.2. Instrumentation

The instruments used include heating and magnetic stirrers, analytical scales, a set of refluxes, a TLC chamber, 254 nm UV light, a TLC scanner, an electrothermal melting point apparatus, ^{1}HNMR 400 MHz, and an FTIR spectrophotometer.

2.3. General Procedure for Synthesis of 5-Fluorouracil Derivatives, 4a – 4g

In the first stage, 5-Fu (0.0812 g, 0.625 mmol) reacted with formaldehyde (37%, 0.9687 mmol, 0.072 mL) and 1.25 mL distilled water in a round bottom flask. Then, it refluxed at 60°C for 6 hours to produce the compound N1-hydroxymethylene-5-fluorouracil (**2**). The results were evaporated at room temperature until the solvent ran out, then dissolved with 12.5 mL of acetone in a round bottom flask [25]. In the second stage, 0.1 mL (0.6875 mmol) of triethylamine was added to (**2**) compound dissolved in acetone. Next, benzoyl chloride derivatives (0.6875 mmol) (**3**) were added to 5 mL of acetone. After the reagent was added, the mixture in the round bottom was stirred at optimum temperatures (ice bath and 40°C) and reaction time for 6-17 hours. The results were evaporated until the solvent ran out, and then distilled water (2.5 mL) and ethyl acetate (7.5 mL) were added to form two phases (organic and water phases). Both phases were washed by adding HCl (pH 3-4) and $NaHCO_3$ (pH 7-8). The remaining organic phase was taken, and Na_2SO_4 was added. Next, the organic phase was evaporated at room temperature, the remaining residue was purified using column chromatography with the eluent hexane: acetone (6:4).

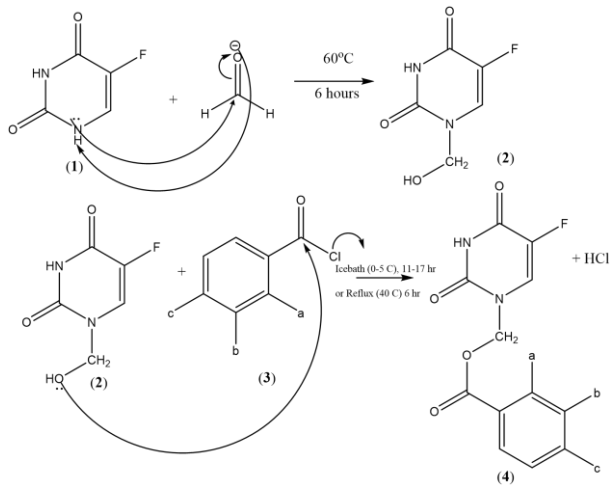


Figure 1. Reaction mechanism of synthesis of 5-Fu derivatives with a benzoyl chloride substituent

All 5-Fu derivatives, **4a – 4g**, were synthesized using the procedures stated above by changing each benzoyl chloride, **3a – 3g** (3-chlorobenzoyl chloride, 2- chlorobenzoyl chloride, 3-nitrobenzoyl chloride, 4- methoxybenzoyl chloride, 4-triflurocarbonbenzoyl chloride, 3,4-dichlorobenzoyl, 4-nitrobenzoyl chloride).

2.4. Structure Identification of the Synthesis Products

Synthesis products of 5-Fu derivatives, **4a - 4g**, were characterized and tested the purity using a TLC scanner (densitometer), melting point apparatus, FTIR, and ^{1}HNMR. ^{1}HNMR (400 MHz) spectra were recorded on an Agilent 400 spectrometer. Chemical shifts (δ) are given in parts per million (ppm) upfield from tetramethylsilane (TMS) as an internal standard. IR spectra (KBr pellets) were recorded on a Shimadzu spectrometer. TLC was carried out using Kieselgel 60 F254 (Merck), while TLC scanners were recorded on a Shimadzu densitometer. Melting points (*mp*) were determined on an electrothermal melting point apparatus Stuart and were uncorrected.

3. Results and Discussion

The mechanism of action and metabolism of 5-Fu has been proposed in two routes: anabolic and catabolic routes. By infusion intake, 5-Fu is converted to active metabolite fluorodeoxyuridine triphosphate (FUTP) or fluorodeoxyuridine diphosphate (FdUDP) to inhibit thymidylate synthase (TS) in the cells. It is also catabolized into dihydrofluorouracil (DHFU) in the liver, which results in smaller metabolites excreted through the kidneys when 5-Fu is consumed as oral intake [2, 4, 5, 6].

In this study, seven compounds of 5-Fu derivatives were synthesized in two stages. The reaction is started by an alkylation reaction of 5-Fu (**1**) with formaldehyde, following the method stated by Tian *et al.* [22], to produce compound **2**, N1-hydroxymethylene-5-fluorouracil. The following reaction is the esterification of compound **2** using benzoyl chloride (Schotten-Baumann reaction), with various substituents at different positions produced by ester-based 5-fluorouracil derivatives **(4)** (Figure 1).

Table 1. The optimized eluent for separation of the 5-Fu derivatives by TLC

5-Fu derivatives	Rs of eluent (hexane: acetone)		Rf (hexane: acetone = 6:4)
	7:3	6:4	
4a	1.04 (tailing)	1.00	0.48
4b	0.70	1.67	0.48
4c	0.59	2.97	0.42
4d	1.41	2.40	0.46
4e	0.94	1.57	0.44
4f	0.66	1.33	0.50
4g	1.17	2.17	0.42

The next step after getting the synthesis product was purification using column chromatography with the eluent hexane: acetone (6:4). The figure of the TLC plate can be seen in Figure 2. Then, the pure compound was examined for its characteristics, including the organoleptic test, the melting point range test, the purity test by TLC, and structure identification using FTIR and ^{1}HNMR.

Compound **(3)**:
3a: a = H, b = Cl, c = H: 3-chlorobenzoyl chloride
3b: a = Cl, b = H, c = H: 2-chlorobenzoyl chloride
3c: a = H, b = NO_2, c = H: 3-nitrobenzoyl chloride
3d: a = H, b = H, c = OCH_3: 4-methoxybenzoyl chloride
3e: a = H, b = H, c = CF_3: 4-trifluoromethylbenzoyl chloride
3f: a = H, b = Cl, c = Cl: 3,4-dichlorobenzoyl chloride
3g: a = H, b = H, c = NO_2: 4-nitrobenzoyl chloride
Compound **(4)**:
4a: a = H, b = Cl, c = H: 1 - (3-chlorobenzoyl oxy methyl)-5-fluorouracil
4b: a = Cl, b = H, c = H: 1 - (2-chlorobenzoyl oxy methyl)-5-fluorouracil
4c: a = H, b = NO_2, c = H: 1 - (3-nitrobenzoyl oxy methyl)-5-fluorouracil
4d: a = H, b = H, c = OCH_3: 1 - (4-methoxybenzoyl oxy methyl)-5-fluorouracil
4e: a = H, b = H, c = CF_3: 1 - (4-trifluoromethylbenzoyl oxy methyl)-5-fluorouracil
4f: a = H, b = Cl, c = Cl: 1 - (3,4-dichlorobenzoyl oxy methyl)-5-fluorouracil
4g: a = H, b = H, c = NO_2: 1 - (4-nitrobenzoyl oxy methyl)-5-fluorouracil

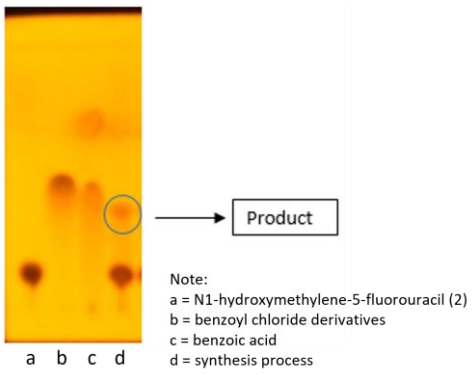


Figure 2. TLC spot of synthesized 5-Fu derivatives

Table 2. The optimized condition at the second step reaction (esterification) on the synthesis of 5-Fu derivatives

5-Fu derivatives	Reaction conditions (temperature)	Time of reaction (hour)
4a	Reflux (40°C)	6
4b	Reflux (40°C)	6
4c	Reflux (40°C)	6
4d	Reflux (40°C)	6
4e	Ice bath (0-5°C)	11
4f	Ice bath (0-5°C)	17
4g	Reflux (40°C)	6

Table 3. The melting point and organoleptic of ester-based 5-fluorouracil derivatives

Synthesis compound	Melting point (°C)	Organoleptic
4a	166 – 167	Crystalline powder
4b	193 – 194	Pale yellow powder
4c	116 – 117	Pale yellow powder
4d	168 – 169	Yellowish-white needle crystal
4e	172 – 174	White needle crystal
4f	184 – 186	White powder
4g	209 – 211	White crystal

3.1. Eluent Optimum

The eluent optimum was analyzed using TLC. The results showed that the value of Rf and Rs optimum of the synthesis products still qualify in a good range, i.e., 0.2 to 0.8 and more than 1.5, respectively. Rs (resolution) is the separation between two analytes on a chromatogram. It can be determined by comparing the distance between the center of the spots and the average diameter of the spots (Table 1).

3.2. The Optimum Temperature and Time of Reflux

Optimization of the reflux time on the second step reaction, esterification, is done to determine the optimum time required to produce the optimum number of synthesis products (Table 2). The first step can result in a 96.44 % yield of N1-hydroxymethylene-5-fluorouracil (**2**). The FTIR spectra of N1-hydroxymethylene-5-fluorouracil (**2**) can be seen in Figure 3.

The optimum temperature and time required for refluxing the mixture at the second step reaction have been studied in this research, and the results are shown in Table 2. The stage 2 reaction temperature is different because the halogen substituent has different electronegativity, which affects the activation of the benzoylation reaction, which will affect the final yield of the reaction. The physical organoleptic results can be seen in Table 3.

Table 4. The yield of ester-based 5-fluorouracil derivatives

Synthesis compound	Yield (%)
4a	61.84
4b	71.51
4c	64.32
4d	69.72
4e	79.27
4f	63.41
4g	72.68

3.3. Characteristics of the Compound in Product Synthesis

The separation of compounds **4a-4g** in the synthesis products using column chromatography has resulted in some groups of fractions. Those fractions produced pure compounds that have single spots at certain Rf were selected for ^{1}HNMR and FTIR tests. The yield of compounds (**4a-4g**) around 61-79% can be seen in Table 4. The melting point test and organoleptic compounds (**4a-4g**) can be seen in Table 3. ^{1}HNMR and FTIR spectra can be seen in Figures 3 and 4.

The information in Table 5 is characteristic of all pure synthesis products, presented in the following order: number of compounds, physical form, melting point (°C), IR spectra (ν_{max}, cm^{-1}), ^{1}HNMR spectra in acetone-d_6, 400 MHz (chemical shift, δ_H, in ppm; number of protons; multiplicity).

Optimization of eluent is done to determine the appropriate eluent for purity test and purification. Based on the value of *Rs* in Table 1, it can be concluded that most of all derivatives of 5-Fu experience better separation when using eluent hexane: acetone (6:4), except for compound **4a**. The results showed that the value of *Rf* and *Rs* optimum of the synthesis products still qualify in a good range, i.e., 0.2 to 0.8 and more than 1.5, respectively.

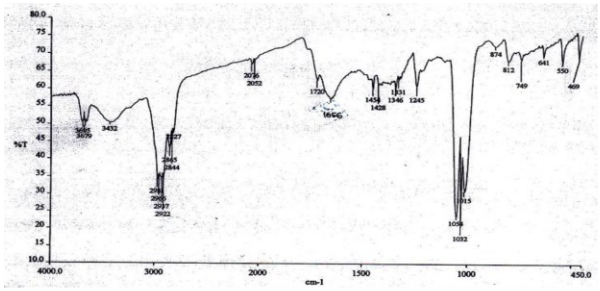
Table 5. Characterization of 5-Fu derivatives using FTIR and ^{1}HNMR

The number of compounds	Characteristic			
	Physical form	MP (°C)	IR Spectra	^{1}HNMR (acetone-d_6, 400 MHz)
4a	Crystalline powder	166-167	1661 cm^{-1} (C=O amide), 1707 cm^{-1} (C=O ester), no O–H signal at 3400 cm^{-1}	10.6 (1H, s); 7.58 (1H, s); 6.0 (2H, s); 8.1 (1H, d); 8.0 (1H, d); 7.72 (1H, d); 8.0 (1H, d)
4b	Pale yellow powder	193-19	1673 cm^{-1} (C=O amide), 1737 cm^{-1} (C=O ester), no O–H signal at 3400 cm^{-1}	8.09 (1H,); 5.97 (2H, s); 7.89 (1H, m); 7.45 (1H, m); 7.58 (1H, m); 7.54 (1H, m)
4c	Pale yellow powder	116-117	1651 cm^{-1} (C=O amide), 1735 cm^{-1} (C=O ester), no O–H signal at 3400 cm^{-1}; 1365 cm^{-1} (-NO_2 aromatic)	7.44 (1H, s); 5.35 (2H, s); 8.71 (1H, d); 8.37 (1H, m); 7.83 (1H, d); 8.47 (1H, s)
4d	Yellowish-white crystalline needles	168-169	1672 cm^{-1} (C=O amide), 1738 cm^{-1} (C=O ester), no O–H signal at 3400 cm^{-1}; 1713 (Ar-ether)	10.64 (1H, s); 8.04 (1H, d); 5.91 (2H, s); 7.03 (2H, d); 7.12 (2H, d); 3.89 (3H, s)
4e	White needle crystals	172-174	1659 cm^{-1} (C=O amide), 1725 cm^{-1} (C=O ester), no O–H signal at 3400 cm^{-1}; 1329 cm^{-1} (polyfluoroalkanes)	10.67 (1H, s); 8.11 (1H, d); 6.01 (2H, s); 8.24 (2H, d); 7.89 (2H, d)
4f	White powder	184-186	1697 cm^{-1} (C=O amide), 1722 cm^{-1} (C=O ester), no O–H signal at 3400 cm^{-1}	10.7 (1H, s); 8.15 (1H, d); 5.98 (2H, s); 7.96 (1H, m); 7.76 (1H, d); 7.74 (1H, d)
4g	White thread-like crystals	166-167	1672 cm^{-1} (C=O amide), 1727 cm^{-1} (C=O ester), no O–H signal at 3400 cm^{-1}; 1352 cm^{-1} (-NO_2 aromatic)	10.59 (1H, s); 8.11 (1H, m); 6.01 (2H, s); 8.29 (1H, m); 8.37 (1H, m); 8.39 (1H, m); 8.31 (1H, m)

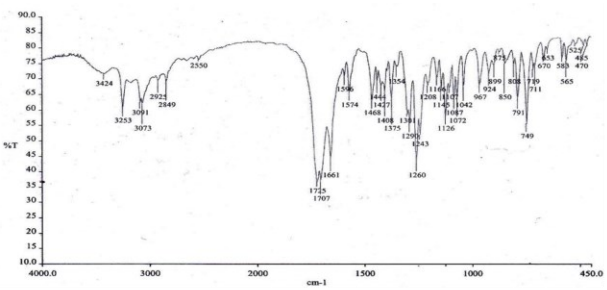
Optimization of the reflux time on the second step reaction, esterification, is done to determine the optimum time required to produce the optimum number of synthesis products. Tian *et al.* [22] found that 6-hour reflux at 60°C is the optimum reaction condition for the first step reaction, alkylation of 5-Fu, to form **2**. That research is continued by Puspaningtyas [37], who optimized the time and temperature of reflux at the second step reaction. This work results in optimum conditions, i.e., 6 hours of reflux at 40°C. However, because the benzoyl chloride substituents used in this study are different from those used by Puspaningtyas [37], it is necessary to optimize the reaction conditions at the second step reaction, in terms of temperature (on ice bath, at room temperature and 40°C) and time (one into 24 hours). Those data are obtained by comparing each spot area between the products and starting material in the chromatogram at each time variation. The time at which the smallest comparison of the spot area between the starting material and product is selected is the optimum temperature and time required for reflux at the second step reaction.

The functional groups of 5-Fu derivatives were identified using FTIR to ensure whether those fractions contained the expected compounds. Interpreting absorption signals that appeared on the FTIR spectrum was then compared with the literature. The important signal that represents the presence of the expected compounds is the appearance of ester carbonyl group absorption at 1750-1730 cm^{-1}. Meanwhile, the signal of -OH at 3400 cm^{-1} in compound **2** is also expected to disappear because it reacts with the benzoyl group to form an ester. The characterization of FTIR spectra can be seen in Figure 3.

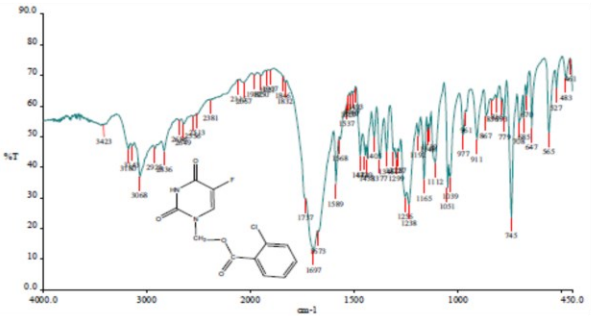
^{1}HNMR spectra of the synthesis product were compared with spectra prediction from the software Chem Office 2008 trial version and the literature. The most important signal in the spectra is at chemical shift 6 ppm, which belongs to the proton signal from $-CH_2-$ and represents the presence of bonding between compound **2** with benzoyl chloride substituent, compound **3**. The chemical shift of the proton in $-CH_2-$ is higher than the literature (4.93) because the carbon in $-CH_2-$ is bonded to two heteroatoms (O and N). According to predictions, the proton signal of –NH groups will appear at a chemical shift of 10 ppm. However, sometimes, the proton signal of the –NH group does not appear due to a weak quality spectrum. Meanwhile, the peak residue from acetone-d_6 usually appears at a chemical shift of 2 ppm. These compounds were also characterized physically, including form and color tests and the melting point range. The characterization of ^{1}HNMR spectra can be seen in Figure 4.



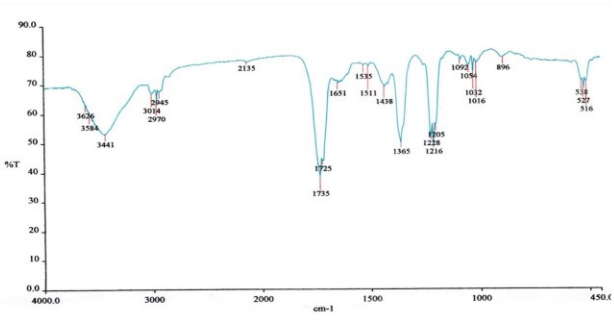
N1-hydroxy-5-fluorouracil (intermediate compounds) (compound 2)



4a



4b



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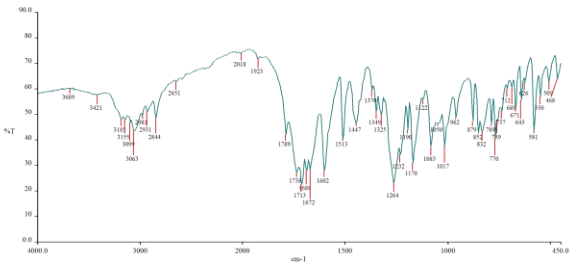

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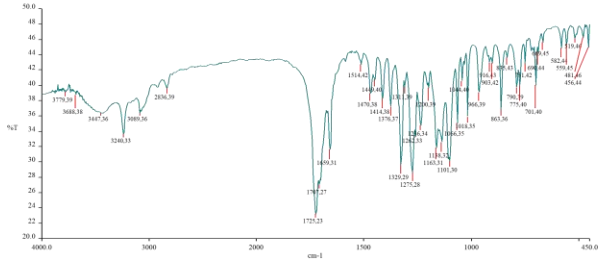

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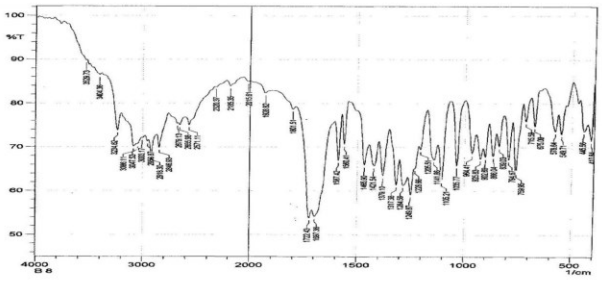
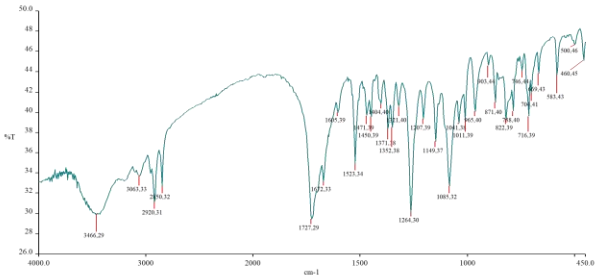


4f



4g

Figure 3. FTIR spectra of 1-hydroxy-5-fluorouracil (intermediate compounds) and 5-Fu derivatives (**4a**, **4b**, **4c**, **4d**, **4e**, **4f**, **4g**)

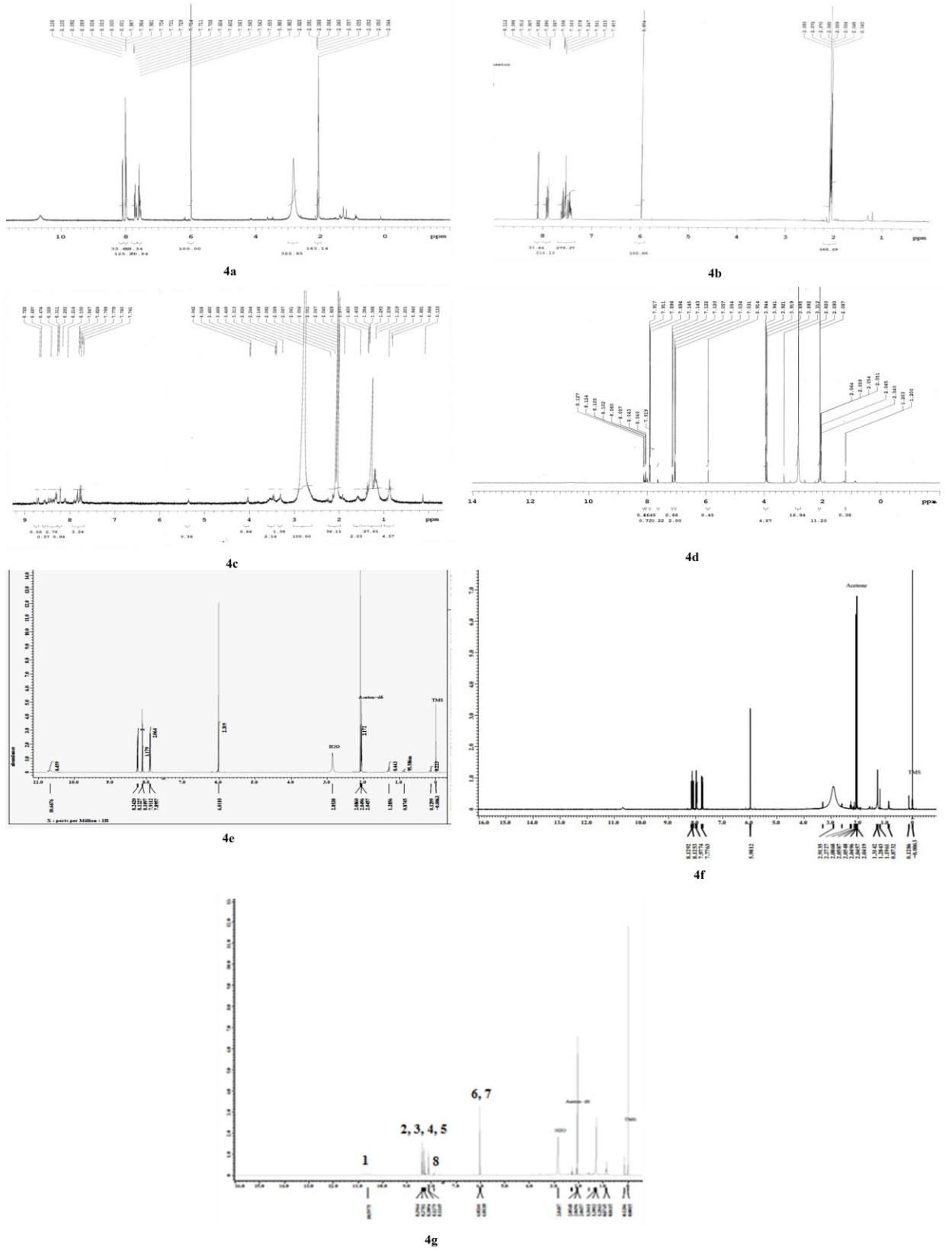


Figure 4. ^{1}H NMR spectra of 5-Fu derivatives (**4a**, **4b**, **4c**, **4d**, **4e**, **4f**, **4g**)

4. Conclusion

The optimum reaction condition of synthesis of novel ester-based 5-fluorouracil derivatives (**4a**, **4b**, **4c**, **4d**, and **4g)** was performed at 40°C for 6 hours. Compounds **4e** and **4f** were optimally synthesized in an ice bath for 11 and 17 hours, respectively. All product synthesis, compound **4a-4g,** was purified using column chromatography and eluted using eluent hexane: acetone (6:4), and the yield of compounds (**4a-4g**) was around 61-79%. This method resulted in some pure compounds (**4a-4g**). Based on the FTIR and ^{1}HNMR data, it can be concluded that all 5-Fu derivatives can be synthesized using the Schotten-Baumann reaction method.

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