

Synthesis of Some Prodrug Compounds Depending on Maleimide Derivatives Method

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Summary: Four different prodrugs were prepared by linking various antibiotic drugs (Ampicillin, Amoxicillin, Ceftriaxone, and Cefotaxime) with 4-maleimidobenzoic (4-(2,5-dihydro-1H-pyrrol-1-yl)benzoic acid), which is firstly converted to 4-(2,5-dihydro-1H-pyrrol-1-yl)benzoyl chloride in the presence of SOCl₂ to form four maleimide derivatives [5a-d]. These derivatives were characterized by FT-IR, 1H NMR, 13CNMR, and C.H.N.S. and then studied for their antibacterial activity against *E. coli* and *S. aureus* bacteria. As well as studied the anti-cancer activities and determined their potential to reduce cell viability in the human breast cancer cell line MCF7. The findings show that the substances created in this study have a promising activity profile in terms of slowing the growth of both cancer cells and the chosen bacteria.

Keywords: Prodrugs, Maleimide, Synergistic effect, MTT assay, and Antibacterial activity.

Introduction

In recent times, significant progress has been seen in the domain of drug design and delivery, with the primary objective of improving therapeutic effectiveness, bioavailability [1], and patient adherence to diverse pharmacological substances [2]. The current medication administration practices often encounter difficulties related to inadequate selectivity, leading to elevated toxicity and drug resistance [3]. These issues stem from the restricted ability of conventional pharmaceuticals to provide precise control over both space and time [4]. The creation of prodrug molecules, which are physiologically inert precursors that undergo enzymatic or chemical changes inside the body to produce the active pharmacological agent, has garnered significant interest in recent times [5]. The failure of infectious disease chemotherapy was caused by the emergence of drug-resistant bacteria. Therefore, there is a critical need for new antimicrobial drugs. One of the most promising approaches to discovering novel, untested molecular targets and their inhibitors appears to be [6]. Prodrugs are strategic modifications of therapeutic medicines that are intended to enhance the pharmacokinetic characteristics of the medication [7,8]. The use of prodrug design has shown its efficacy in enhancing the pharmacological characteristics of a compound [9], rendering it more suitable for therapeutic applications. Consequently, this approach has gained significant traction in the field of drug discovery, encompassing a diverse range of medical conditions [10]. Enhancing therapeutic efficacy and

refining the physicochemical attributes of a pharmaceutical compound [11], such as its lipophilicity and aqueous solubility, may be accomplished by implementing targeted structural alterations [12]. Additionally, altering the route of drug administration can also contribute to these improvements [13]. The capacity to adapt has facilitated the advancement of prodrug development in several therapeutic domains, including pain control, cancer therapy [14], and central nervous system illnesses [15]. One significant benefit associated with prodrugs pertains to their capacity to augment medication delivery [16]. This research endeavors to explore the synthesis of a series of prodrug compounds based on maleimide derivatives.

Previous studies have shown that cyclic imides and their derivatives have medicinal value for biological activities [17] such as antibacterial [18], analgesic, and antifungal effects, and anticancer [19, 20]. Maleimides are now being studied as potential drug [21] candidates because they are important pharmacophores with a variety of biological activities [22], including antibacterial properties [23][24]. Notably, the naturally occurring maleimide framework found in himanimide and its synthetic analogues have demonstrated promising antimicrobial activities [25] [26]. Maleimide, a heterocyclic molecule with an unbroken imide ring and the generic structural formula (-CO-N(R)-CO-), exhibits hydrophobic and neutral properties and has the ability to

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readily traverse cell membranes[27] [28]. The investigation of compounds that include maleimide structures is a common practice within chemical probes[29] the realm of biomaterials research, and organic dyes[30]. Moreover, different derivatives maleimide have been artificially prepared and have shown remarkable antifungal, antibacterial [31] and herbicidal activities[32]. The biological activity of maleimides[33] is primarily attributed to their reactivity with free thiol groups of cysteine residues found in enzymes[34]. Maleimides are known to be potent Michael acceptors for thiol and amine nucleophiles[35]. The goal of the current study was to synthesize novel maleimide-drug derivatives by condensation with different drugs [36]. Drug molecules that are covalently joined to polymer molecules form polymer-based prodrugs, which are used as drug delivery systems [37]. There are numerous bioactive applications for maleimide polymer drugs [38]and [39]. In the field of cancer research of maleimide, antibody-drug conjugates (ADCs) have emerged as a novel class of therapies[40]. These structures possess the remarkable ability to selectively target antigens, similar to antibodies, while also exhibiting the powerful cytotoxic effects often associated with small-molecule medicines[41].

Experimental

Material

Analytical-grade reagents and solvents from reputable suppliers, including Sigma-Aldrich (Germany),

Fluka (Switzerland), and Thomas Baker (UK), were used in the study. The drugs used in the study were obtained from SDI-Samarra Company (IRAQ).

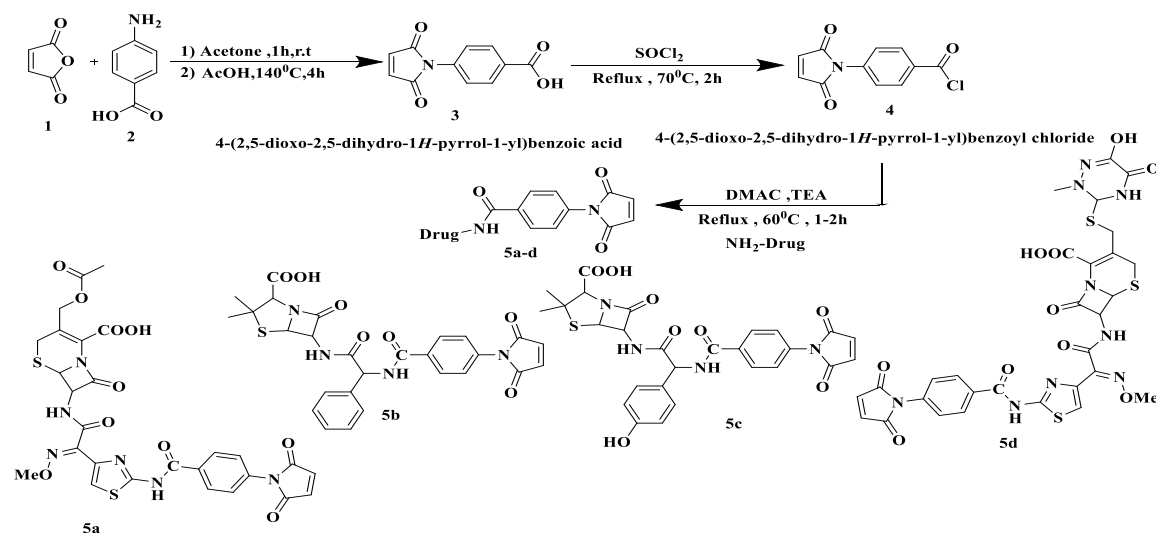
Instrumental analysis

A SMP30 melting point apparatus (United Kingdom) was used to calculate the melting points of the samples. A Bruker Tensor II FT-IR spectrophotometer (Germany) was used to record Fourier Transform Infrared (FT-IR) spectra between 400 and 4000 cm^{-1} . Microelemental analysis was carried out using the Euro EA3000 Elemental Analyzer from (Italy) to estimate the content of C, H, N, and S. The ^1H NMR spectra were recorded using a Varian INOVA 400 MHz NMR spectrometer in dimethyl sulfoxide (DMSO- d_6), while the ^{13}C NMR spectra were recorded at a frequency of 125.59 MHz in DMSO- d_6 . The chemical shifts were reported in δ units (ppm). (University of Tehran, Iran).

Synthesis of 4-maleimidobenzoic acid (3)

A mixture of p-aminobenzoic acid (**2**) (20.55 g, 0.15 mol) in 100 mL of dry acetone was slowly added to a solution of maleic anhydride(**1**) (14.7 g, 0.15 mol) in 50 mL of dry acetone. The resulting mixture was stirred continuously at room temperature for 2 hours until a yellow precipitate of 4-maleimidobenzoic acid(**3**) was formed ($R.f = 0.46 / 1\text{hexane} : 3\text{ethylacetate}$). The precipitate was filtered and washed with distilled water several times. The obtained product was recrystallized from hot ethanol to give pure compound (**3**) in a 90% yield with a melting point of 226-228 $^{\circ}\text{C}$.

Preparation methods



Scheme-1: Synthesis route of compounds (**5a-d**)

FT-IR analysis showed peaks at 3500-2500 cm^{-1} (COOH), 3098 and 3003 cm^{-1} (=C-H, maleimide and aromatic), 1709 and 1693 cm^{-1} (CO-N-CO- and C=O carboxylic acid), 1606 cm^{-1} (C=C, maleimide), and 1553-1473 cm^{-1} (C=C, aromatic).

Synthesis of compound N-[4-(Chlorocarbonyl) phenyl] maleimide (4)

The synthesis of the acid chloride from compound (3) involved refluxing 4.29 g (0.011 mol) of compound (3) in 20 mL of thionyl chloride at 70 °C for 2 hours. The resulting solution was then evaporated under reduced pressure, and 10 mL of carbon tetrachloride (CCl_4) was added to the residue to remove any remaining solvent. The final product was obtained as light yellow crystals with a 90% yield and a melting point of 172-174 °C (compared to a literature value of 167-170 °C). The FT-IR spectrum of the product showed peaks at 3108 and 3081 cm^{-1} (indicating C-H stretching), 1769 cm^{-1} (COCl stretching), 1718 cm^{-1} (CONCO stretching), 1597-1455 cm^{-1} (aromatic C=C stretching), and 1371 cm^{-1} (C-N stretching).

Maleimide-Drug Derivative Synthesis (5a-d)

In 10 mL of dimethyl acetamide and 1 mL of TEA, separate solutions of 4 mmol of five different medications (namely, cefotaxime, ampicillin, amoxicillin, and ceftriaxone) were made. Separate additions of each of these solutions were made to a stirred solution of compound (4) (1 g, 4 mmol), which was then gradually heated for two hours at 60 °C. Using TLC, the reaction was observed. The solution was added to crushed ice after the reaction was finished, allowed to sit for an hour, filtered, and further crystallized from 75% ethanol.

Cefotaxime-4-Maleimidobenzamide (5a)

80% yield, light brown color, and m.p. = 218 °C. 3500-2496 (COOH), 3308, and 3210 in the ATR-FTIR (cm^{-1}) spectrum (-NH, Amide). 3190 (=C-H, aromatic rings and maleimide), 2978-2882 (C-H, sp³), 1781 (C=O, beta-lactam), and 1746 (C=O, ester) are some examples of C-H compounds. 1714 (C=O amide and C=C), 1666 (C=O amide and C=C), 1653, and 1589 (C=N-OMe and C=N thiazole ring), respectively. (C=C, aromatic) 1538-1442.

¹HNMR (500 MHz, DMSO-*d*₆, δ ppm) : 2.63 (s, 3H, -CO-CH₃), 3.74 (s, 2H, methylene protons), 3.81

(s, 3H, =NO-CH₃), 5.04 (s, 2H, methylene of ethanoic ester), 5.16 and 5.92 (2 H, beta lactam), 6.89 (s, 2H, CH=CH), 6.731 -7.999 (m, 5H, Ar-H), 9.04 and 9.675 (2H, Amides), 13.13 br (s, 1H, COOH). **C.H.N.S** Elemental analysis for C₂₇H₂₂N₆O₁₀S₂: Calculated % (49.54, 3.39, 12.84, 9.80) / Found % (48.22, 3.11, 11.35, 9.20).

¹³C NMR (125 MHz, δ , ppm): 25.52 (1C, CO-CH₃), 29.45 (1C, methylene), 63.10 (2C, beta-lactam ring), 72.34 (2C, methoxy and methylene ester), 113.46 -137.75 (Ar-C carbons), 144.11 (2C, C=C of Maleimide ring), 164.67 and 166.00 (3 C, beta lactam CO and 2C-NH-), 168.87 (1C, CO-NH-), 169.97 (2C, N-C=O of maleimide ring), 173.40 (1C, COOH).

Ampicillin-4-maleimidobenzamide (5b)

80% yield, light pink color, m.p. of 191-193 °C. ATR-FTIR (cm^{-1}): 3500-0624 (COOH), 3304, and 3276 (-NH, amide). A few examples of compounds with the formula C-H are: 3080 (=C-H, aromatic rings and maleimide), 2970 and 2917 (C-H, methyl and methylene groups), 1758 (C=O, beta-lactam), 1712 (CO-N-CO, imide), 1658 (C=O, amide and carboxyl group), 1633 (C=C, maleimide), and 1590-1451 (C=C, aromatic).

¹HNMR (500 MHz, DMSO, δ ppm) figure: 1.49 (s, 6H, 2-CH₃), 4.12 (s, 1H, >CH-COO), 4.98 and 5.41 (2H, beta-lactame) 5.80 (s, 1H, >CH-CO), 7.19 (s, 2H, CH=CH), 7.40-7.93 (m, 9H, Ar-H), 9.39 and 10.07 (2H, 2 ArCO-NH-), 13.03 (br, s, 1H, -COOH). **C.H.N.S** Elemental analysis Chemical Formula: C₂₇H₂₄N₄O₇S: Calculated % (59.12, 4.41, 10.21, 5.85) / Found % (58.10, 4.25, 9.59, 5.32).

¹³C NMR (125 MHz, δ , ppm): 35.02 (2C, 2CH₃), 60.19 (1C, ph-C<), 65.69 (1C, C< (CH₃)₂), 68.21 (2C, beta-lactam ring), 75.02 (1C, >C-COO) 121.68 -140.37 (C-Ar), 150.74 (2C, C=C of Maleimide ring), 164.84 and 167.14 (3 C, beta lactam CO and 2C-NH-), 167.86 (1C, CO-NH-), 174.98 (2C, N-C=O of maleimide ring), 175.46 (1C, COOH).

Amoxicillin-4-maleimidobenzamide (5c)

85% yield, yellow color, m.p. of 148-150 °C. Figure 8 of the ATR-FTIR shows the following values: br, 3500-2500 (-OH carboxylic and phenol), 3305, 3270 (-NH amide), 3067 (=C-H, Ar-H, Maleimide), 2976 (C-H, aliphatic), 1775 (C=O, beta-lactam), 1710 (CO-N-CO), 1649 (C=O carboxylic acid and amide groups), 1611 -1450 (C-N stretching).

¹HNMR (500 MHz, DMSO, δ ppm) figure: 1.13 (s, 6H, 2-CH₃), 4.12 (s, 1H, >CH-COO), 4.64 (s, 1H, phenol-CH-CO), 5.12 and 5.77 (2H, beta-lactame), 6.71 (1H, Ph-OH), 7.16 (s, 2H, CH=CH), 7.24-7.91 (m, 8H, Ar-H), 10.13 and 10.43 (2H, 2 ArCO-NH-), 12.12 (s, 1H, -COOH). **C.H.N.S** Elemental analysis for Chemical Formula: C₂₇H₂₄N₄O₈S: Calculated % (57.44, 4.28, 9.92, 5.68) / Found % (57.15, 4.20, 9.66, 5.27).

¹³C NMR (125 MHz, δ , ppm): 35.02 (2C, 2CH₃), 58.00 (1C, ph-C<), 65.01 (2C, beta-lactam ring), 73.31 (1C, C< (CH₃)₂) 76.65 (1C, >C-COO) 121.68 - 140.37 (C-Ar), 150.74 (2C, C=C of Maleimide ring), 164.84 and 167.14 (3 C, beta lactam CO and 2C-NH-), 167.86 (1C, CO-NH-), 174.94 (2C, N-C=O of maleimide ring and 1C, COOH).

Ceftriaxone-4-maleimidobenzamide (**5d**)

A light yellow solid with a melting point of 191–193 °C and an 80% yield is the end result. The results of an ATR-FTIR analysis show absorption peaks at wavelengths of about 3500-2551 cm⁻¹ (COOH), 3273 cm⁻¹ (-NH, amide), 3153 cm⁻¹ and 3013 cm⁻¹ (=C-H, Ar-H and HC=CH), 2944 cm⁻¹ (C-H, methyl), 1767 cm⁻¹ (C=O, beta-lactam), 1713 cm⁻¹ (CO-N-CO), 1667 cm⁻¹ and 1632 cm⁻¹ (O-Me stretching).

¹HNMR (500 MHz, DMSO-*d*₆, δ ppm): 2.78 (s, 3H, N-CH₃), 3.06 (s, 2H, S-CH₂-), 3.58 (s, 3H, NO-CH₃), 3.74 (s, 1H, triazin -CH-), 4.37 (d, 1H, beta-lactam), 5.12 (s, 1H, -OH), 5.76 (d, 1H, beta-lactam), 6.72 (s, 1H, thiazole ring), 7.20 (s, 2H, CH=CH), 7.44-8.20 (m, 4H, benzene ring), 9.58 and 10.75 (2H, Amides) 12.15 (s, 1H, COOH). **C.H.N.S** Elemental analysis Chemical Formula: C₂₉H₂₅N₉O₁₀S₃: Calculated % (46.09, 3.33, 16.68, 12.73) / Found % (44.95, 3.50, 16.14, 12.44).

¹³C NMR (125 MHz, δ , ppm): 25.52 (1C, -S-CH₂-), 29.45 (1C, -S-CH₂-), 33.33 (1C, N-CH₃), 72.23 (2C, beta-lactam ring), 103.71-136.80 (C-Ar), 137.75 (1C, C-OH), 144.11 (2C, C=C of Maleimide ring), 164.67 (1C, CO triazine), 166.00 and 166.68 (3 C, beta lactam CO and 2C-NH-), 168.87 (1C, COOH) CO-NH-), 169.97 (2C, N-C=O of maleimide ring).

Cytotoxic assay

The synthesized compounds were evaluated for in vitro MTT reduction against a human breast cancer cell line. The cells were cultured in RPMI 1640 media containing 10% fetal bovine serum at 37° C, 5% CO₂, and 96% humidity (FBS). To allow cells to

adhere to the bottom of the wells after numerous subcultures, cells were dispensed in 96-well plates at a ratio of 1,000 cells per 100 L of culture medium and incubated for 24 hours at the same temperature.

After removing the culture media, 100 L of the same medium were added to each well in triplicate. The tested substances were present in this medium in a range of concentrations (500, 400, 300, 200, 100, 50, and 25 g/ml). Plates were then incubated under the same conditions for an additional three days.

The final column of the experiment's plate, which contained 1000 cells in 100 μ L of growing media, represented the control group. After three days of incubation, the drug-containing medium was removed, and MTT solution (4 mg/mL in PBS) was added to each well to measure cell survival. The same conditions persisted for an additional three hours of incubation on the plates. Then, each well received 100 μ L of DMSO, and the plates were lightly shaken to disperse the formazan crystals. With the aid of an ELISA plate reader, the absorbance of each well was determined at 540 nm. The formula used to determine the percentage of growth inhibition is 100 (ODtest ODcontrol) 100, where OD test is the mean absorbance of treated cells and ODcontrol is the mean absorbance of the negative control. To calculate the IC₅₀ values for each cell line, dose-response curves were used. The cell survival rate of the control group was taken to be 100%. With GraphPad Prism 6, an unpaired t-test was used to statistically analyze the results. The average plus or minus standard deviation of three independent measurements was used to represent the results.

Results and Discussions

By using TEA as a catalyst to combine 4-maleimidobenzoyl chloride with a variety of medications (Cefotaxime, Ciprofloxacin, Amoxicillin, and Ceftriaxone), the new maleimide-based drug units were produced in high yields. Some of the prepared monomers' [5a-d] physical characteristics are shown in Table-1.

Spectral analysis:

In its FT-IR spectrum, the Comp. [1] does not show an absorption peak for N-H. Instead, it is noted that there are two absorption bands at 1709 cm⁻¹ and 3098 cm⁻¹, which, respectively, correspond to the stretching of (CO-N-CO) and (H-C=C-H). Maleimide

has been successfully cyclodehydrated as evidenced by the broad band of absorption from benzoic acid's (COOH) in the range of 3500–2551 cm⁻¹. The broad hydroxyl group absorption band in the FT-IR spectrum of comp. [2] vanishes, and a new band (1769 cm⁻¹) corresponding to (COCl) emerges at a higher frequency.

While the (COCl) peak vanishes in the [5a-d] FT-IR spectra and a distinctive (CO-N-H-) amide absorption appears in the (3308-3200 cm⁻¹) cm⁻¹ range. Maleimide carbonyl groups are visible at 1714-1709 cm⁻¹, and beta lactam carbonyl is responsible for

the distinctive stretching bands at 1758-1781 cm⁻¹. Additionally, a wide range of carboxylic group absorption of drugs is seen between 3500 and 2500 cm⁻¹.

The prepared monomers' ¹H-NMR spectra exhibit significant signals for beta lactam protons at 4.37–5.92 ppm and maleimide (CH=CH) protons at 6.718–7.182 ppm. The group of drugs that contain carboxylic acids also exhibits a broad and weak signal between 12.12 and 13.13 ppm.

Table-1: Physical properties of prepared monomers (5a-d).

Comp.	Color	Yield (%)	m.p. °C	R _f
M1	light Brown	80%	= > 218 dec	0.551 hexane:3ethylacetate
M2	light pink	80%	191- 193 °C	0.64 1hexane :3ethylacetate
M13	yellow,	85%	148– 150 °C	0.80 1hexane : 3acetone
M4	Light yellow	80%	191-193 °C	0.75 1hexane : 3acetone

m.p=melting point , R.F=Retention time

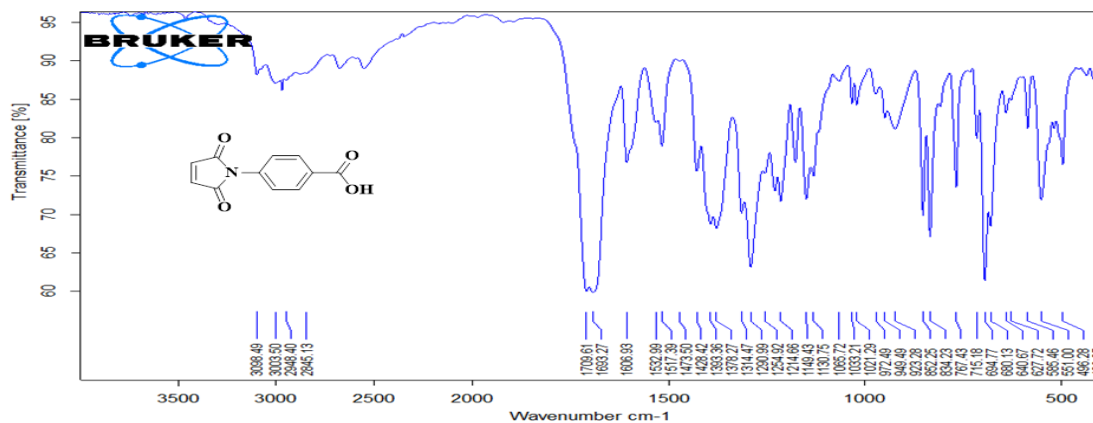


Fig. 1: FT.IR of comp.3

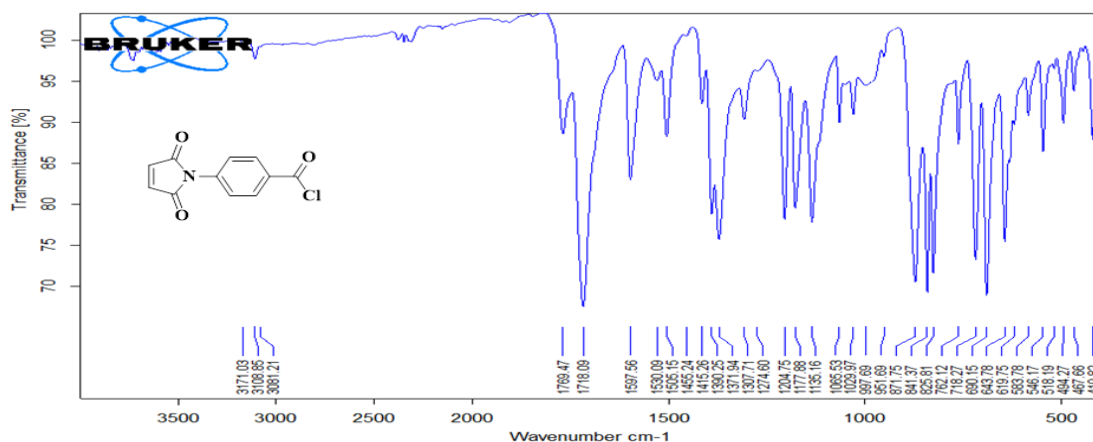


Fig. 2: FT.IR of comp.4

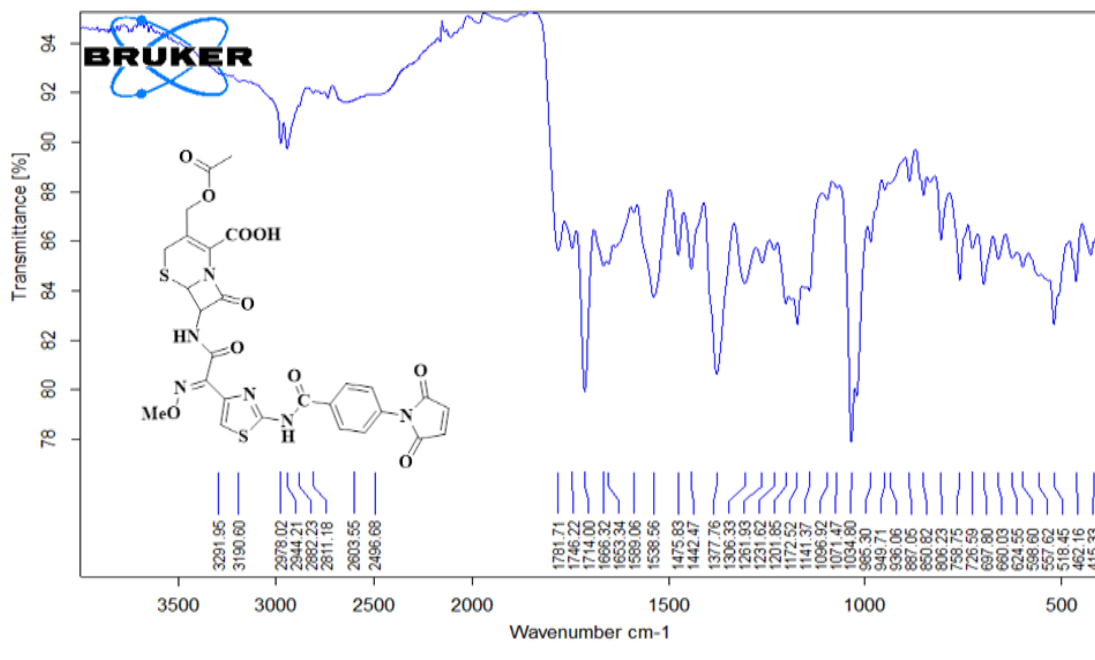
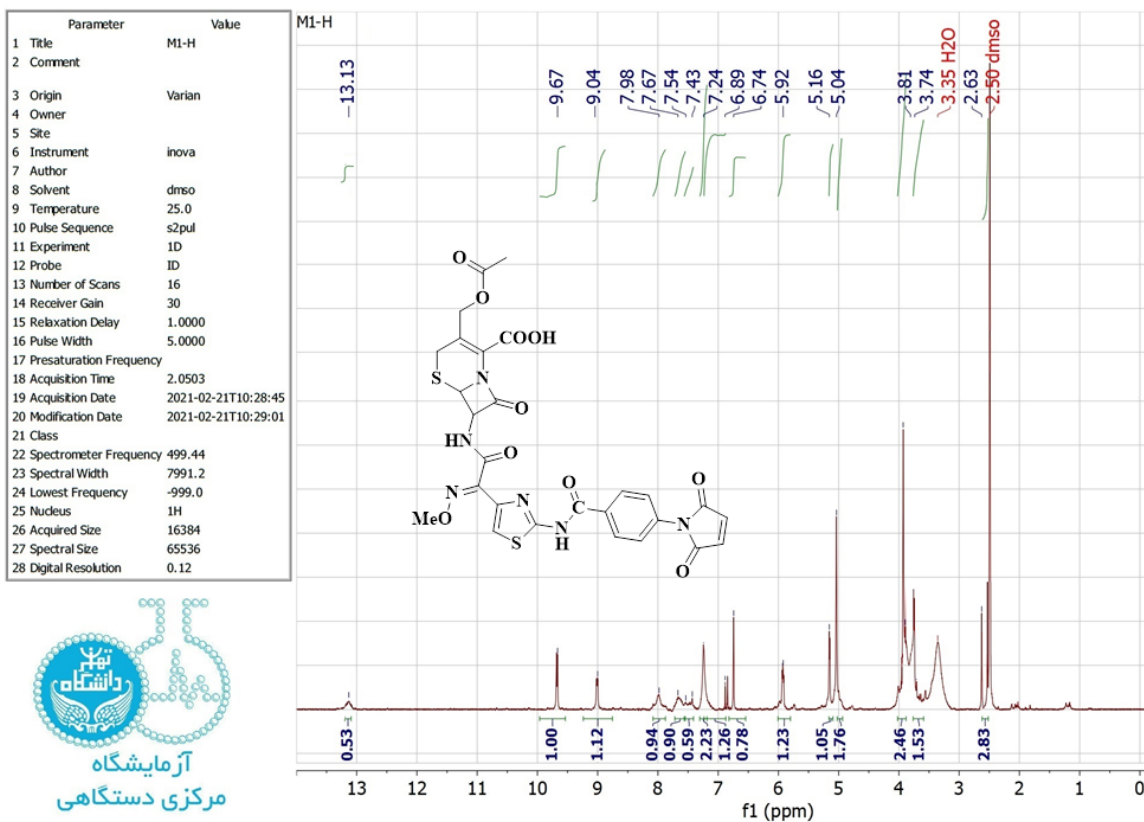
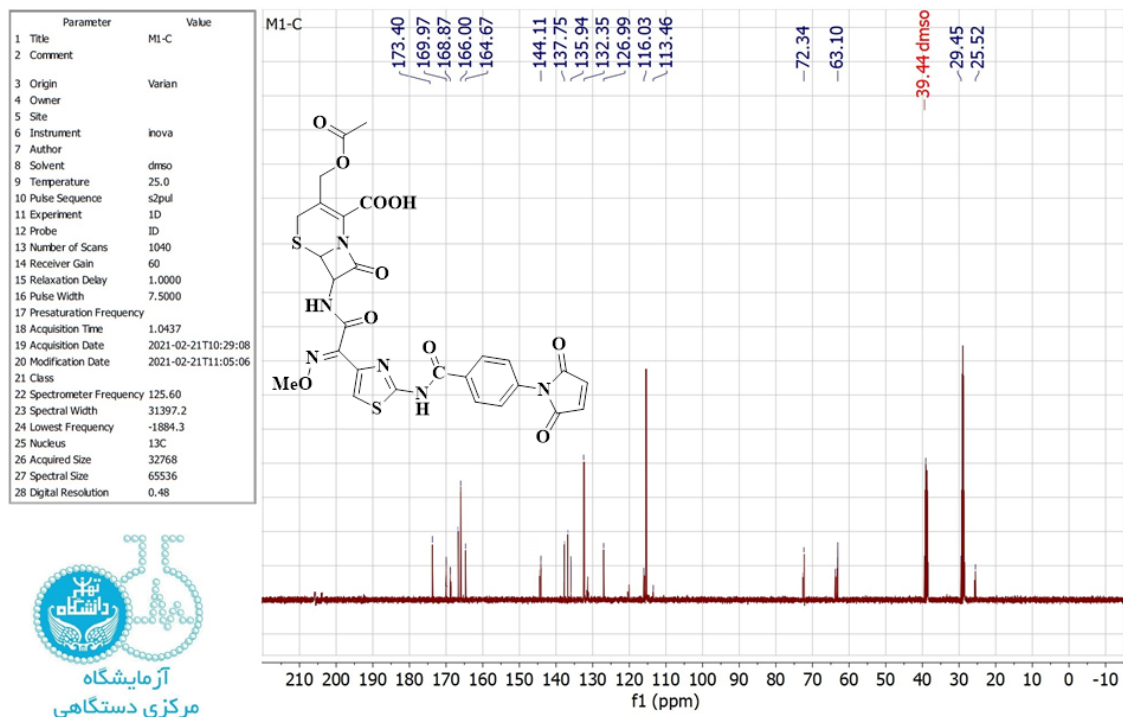
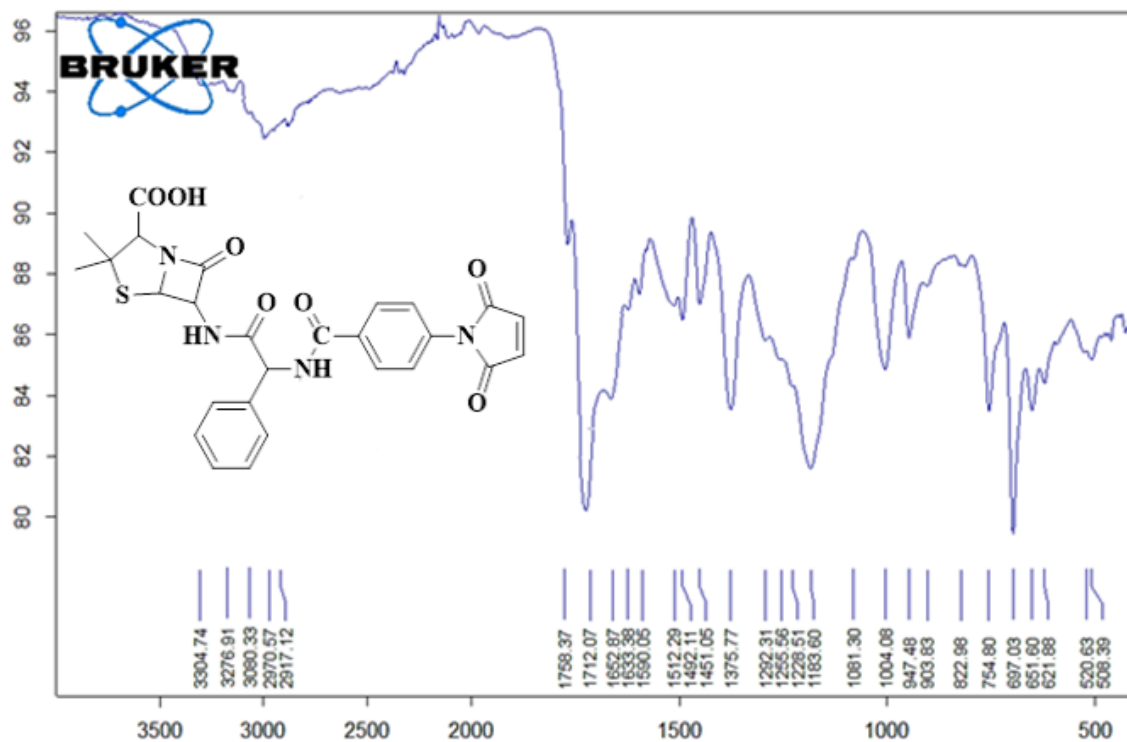


Fig. 3: FT-IR of 5a.

Fig. 4: ¹H NMR of 5a.

Fig. 5: ^{13}C NMR of **5a**.Fig. 6: FT-IR of **5b**.

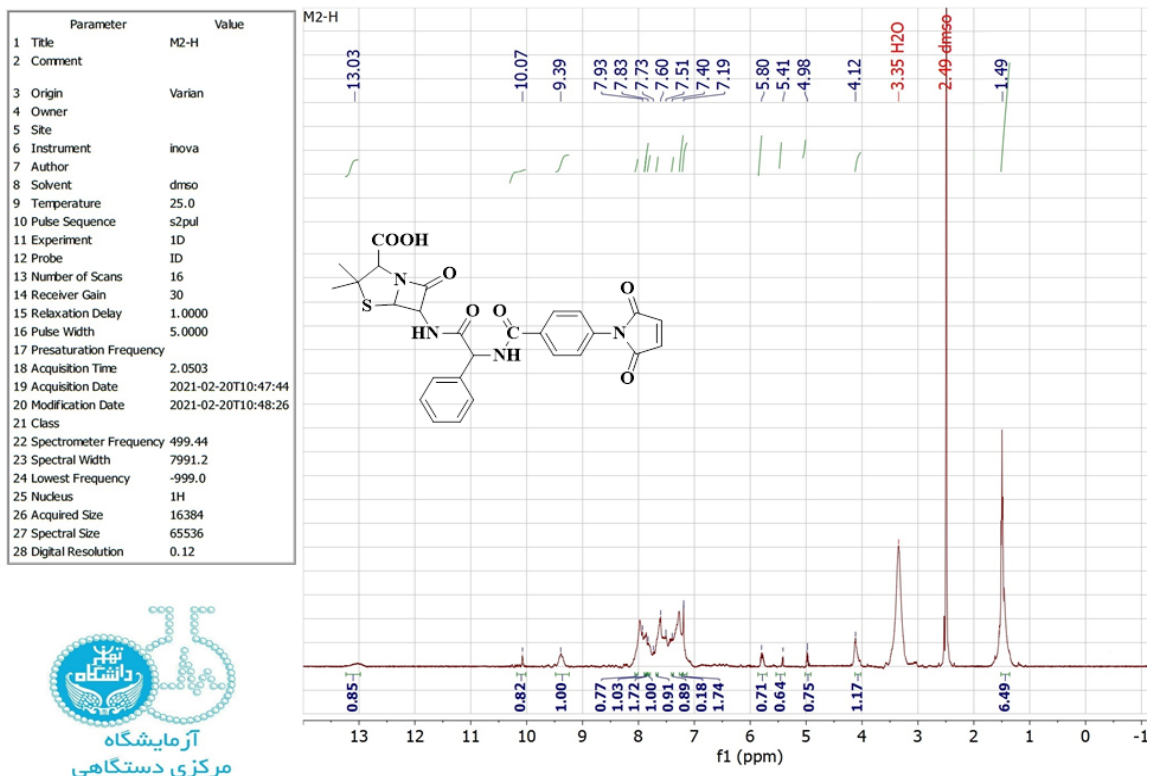


Fig. 7: ¹HNMR of 5b.

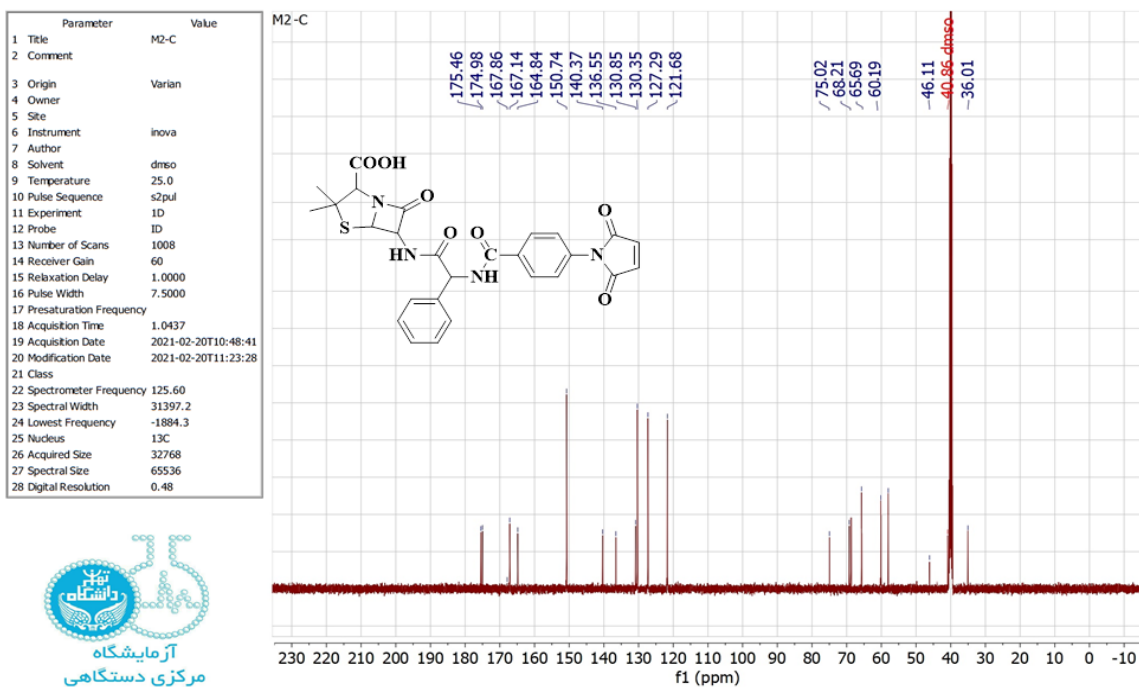


Fig. 8: ¹³CNMR of 5b.

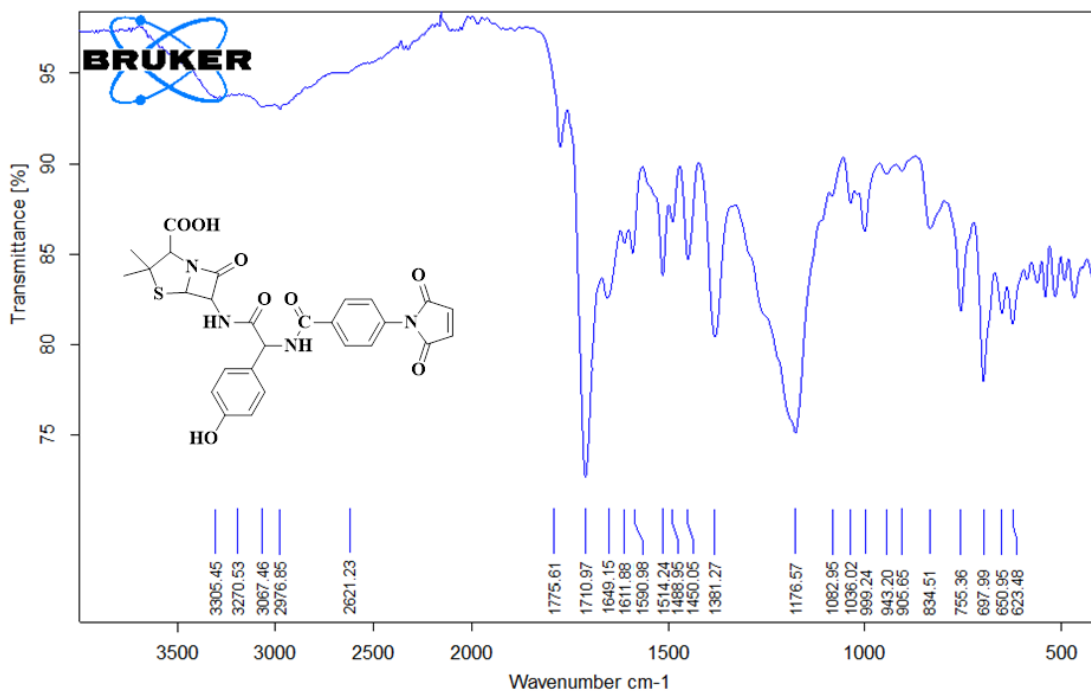


Fig. 9: FT-IR of 5c.

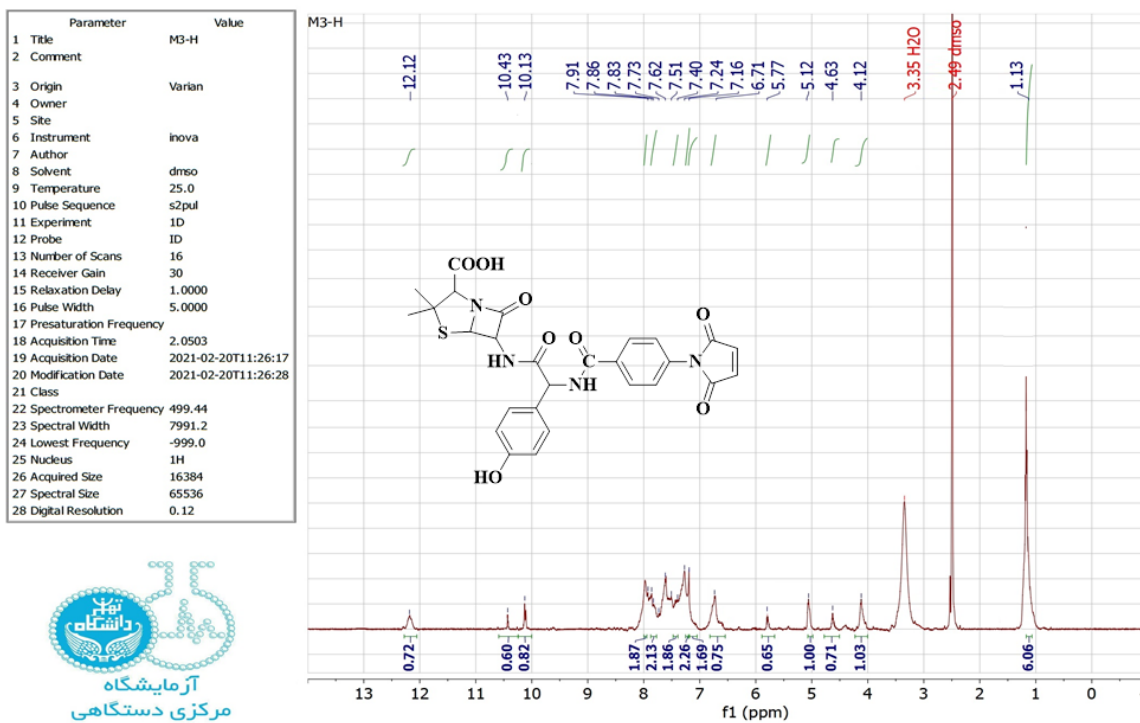


Fig. 10: ¹HNMR of 5c.

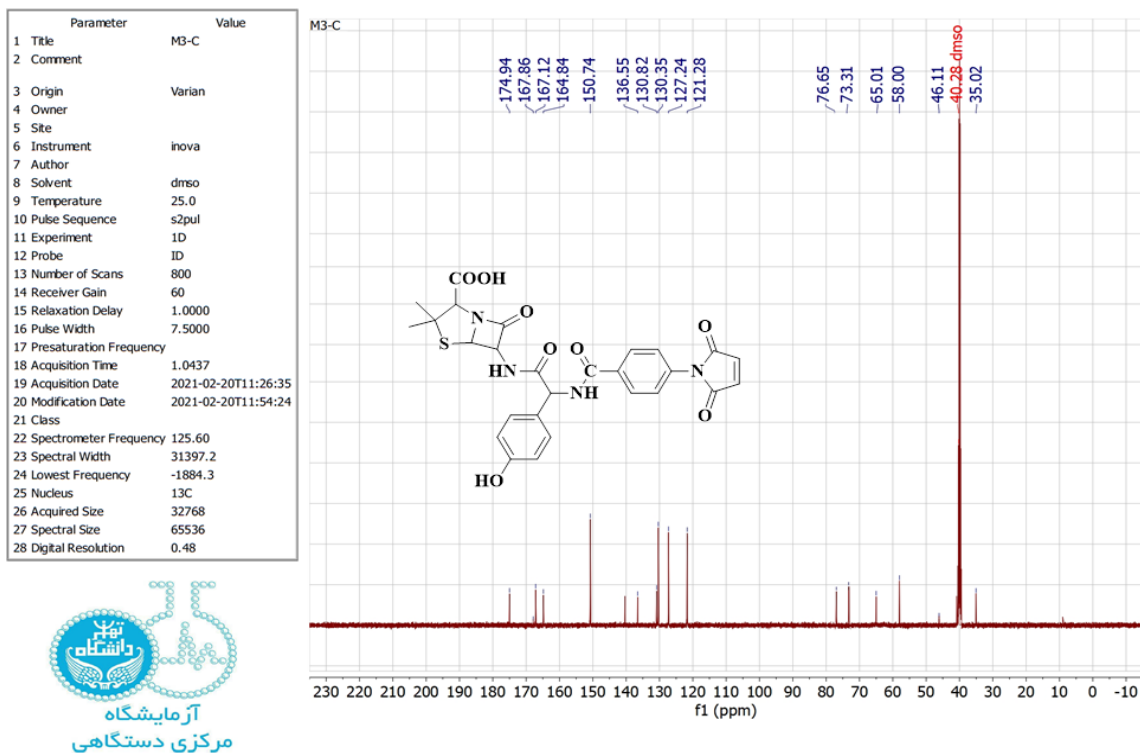


Fig. 11: ¹³CNMR of 5c.

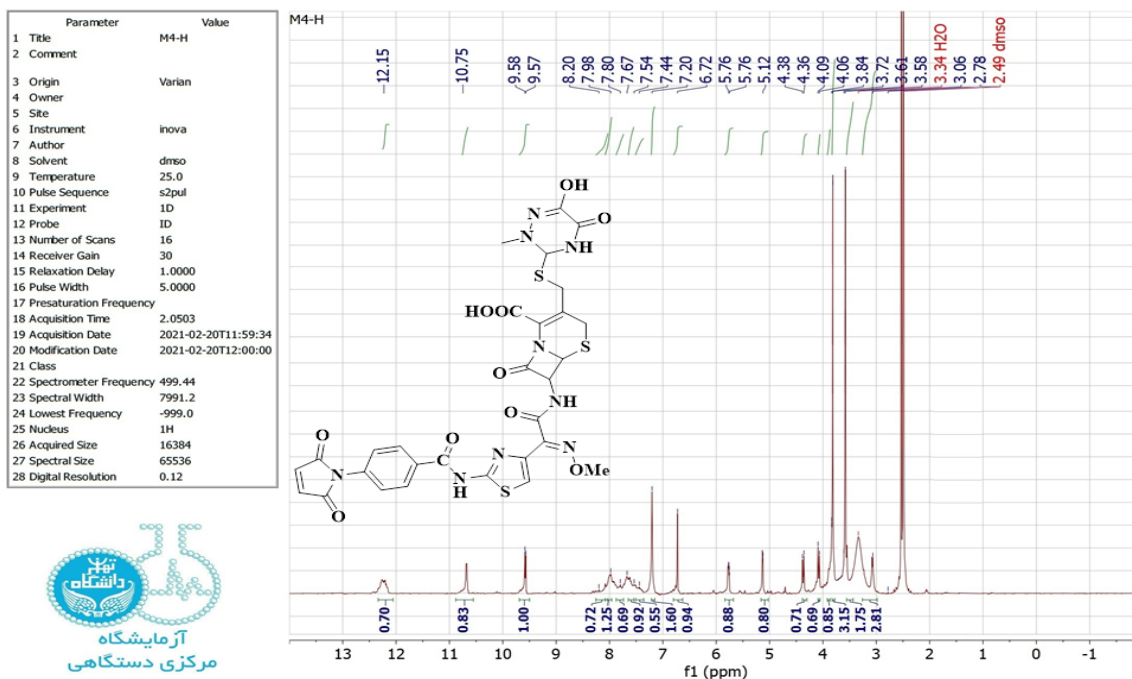
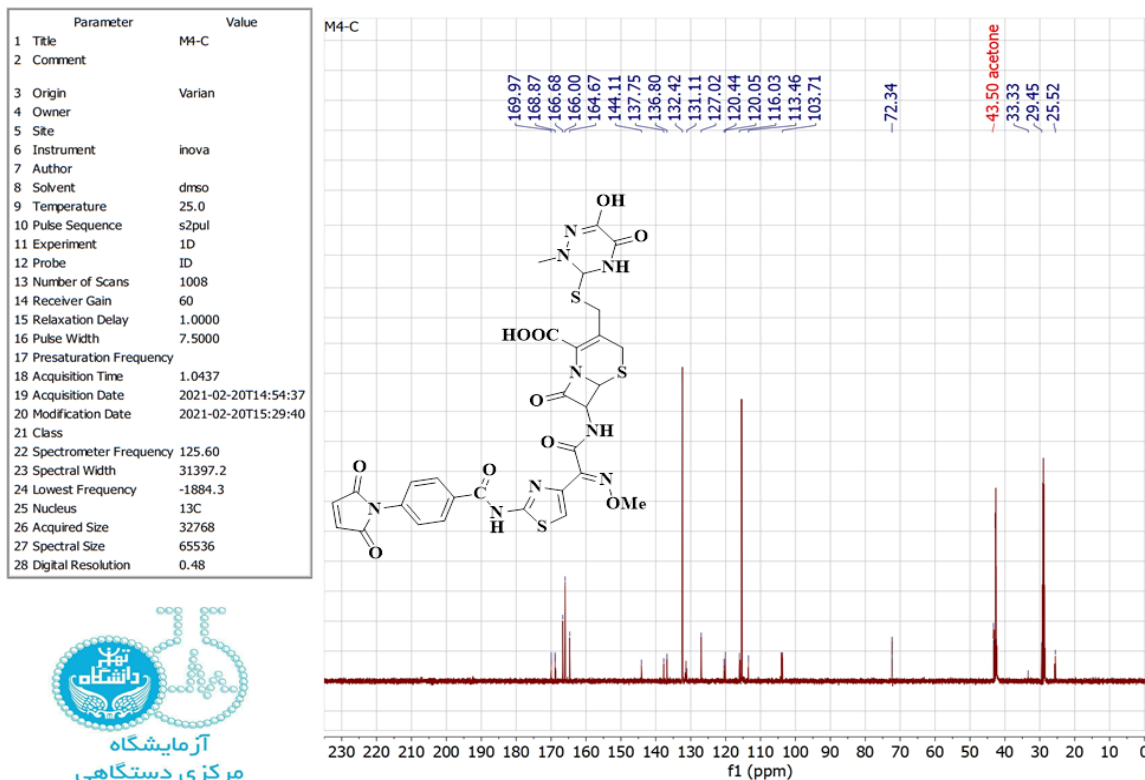


Fig. 12: ¹HNMR of 5d.

Fig. 13: ^{13}C NMR of **5d**.

The Antibacterial Activity

The antibacterial activity of the synthetic compounds was evaluated using the disk-diffusion method against pathogenic strains of *Escherichia coli* (ATCC 8739) and *Staphylococcus aureus* (ATCC25923). For comparison, a solution was made in 1 mL of DMSO with 0.05 mg of each substance and each bound drug. The DMSO screening of the negative control did not show any bacterial growth inhibition. All synthetic compounds' antibacterial properties, along with the drugs they were loaded with, were listed in (Table 2)

Table-2: Antibacterial activity of compound 5a-d (C = 0.05 mg/mL).

Zone of inhibition (mm) for the tested microorganisms is ± 1		
Samples	<i>S. aureus</i>	<i>E. coli</i>
5a	30	34
5b	32	42
5c	28	34
5d	38	30
Drug		
Cefotaxime	20	38
Ampicillin	25	15
Amoxicillin	33	30
Ceftriaxone	35	20
DMSO	0	0

.Gram-negative *E. coli* antibacterial activity results show that **5b**, **5c**, and **5d** compounds have higher activity, with inhibition zones of 42, 34, and 30 mm, respectively, in comparison to Ampicillin, Amoxicillin, and Ceftriaxone, which have inhibition zones of 15 mm, 30 mm, and 20 mm, respectively. **5a** has an antagonistic effect, with a 34 mm inhibition zone, in comparison to Ceftriaxone (38 mm).

The prepared derivatives are showing efficient antimicrobial activity against *S. aureus*, highest diameter of inhibition for 5a, 5b and 5d (30, 32 and 38 mm) than Cefotaxime, Ampicillin and Ceftriaxone (20, 25 and 35 mm). While, **5c** shows an antagonistic effect as result of decreases the diameter of inhibition.

The neutral and lipophilic properties of the maleimide moiety enables it to easily penetrate the cell membranes of bacteria, making it an effective antibacterial agent. Additionally, gram-negative bacteria have an outer lipid-rich membrane that can be easily penetrated by the maleimide, making it more effective against *E. coli*

than gram-positive bacteria like *Staphylococcus aureus*, which have a thicker peptidoglycan layer in their cell walls.

Cytotoxicity Assay

Using the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay, Maleimides were produced, and their cytotoxic effects on the human breast cancer cell line (MCF7) were investigated. The outcomes were compared to the untreated control. The results demonstrate that, in comparison to the control, all of the derivatives inhibited cell proliferation in a concentration-dependent manner.

Compound **5d** inhibited the majority of cells, killing 75% of them at a concentration of 500 ng/mL. At a concentration of 100 µg/mL, 51% of the cells died, and the Inhibitory Concentration value (IC₅₀) was 200.07 µg/mL. (Fig. 16). In contrast, at 100 µg/mL for **5b**, 43% of cells died, and the inhibitory concentration value (IC₅₀) was 220.19. Cell inhibition was maximum at 500 µg/mL for **5a** (68%), **5b** (66%), and **5c** (72%). (Fig. 14). All of the synthesized products, according to the results, were successful in slowing the growth of cancer cells, demonstrating that they might be a workable and promising method for creating a clinically useful drug delivery system for the treatment of breast cancer.

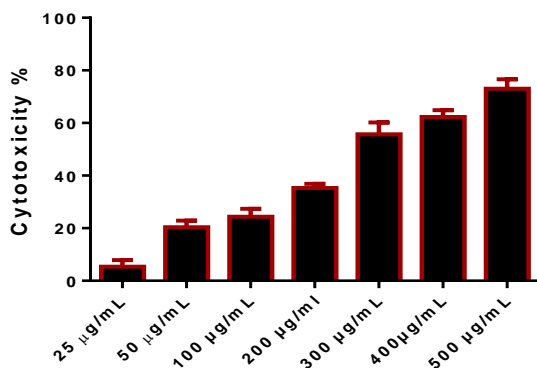


Fig. 13: Cytotoxic result of **5a** IC₅₀=311.13 µg/ml.

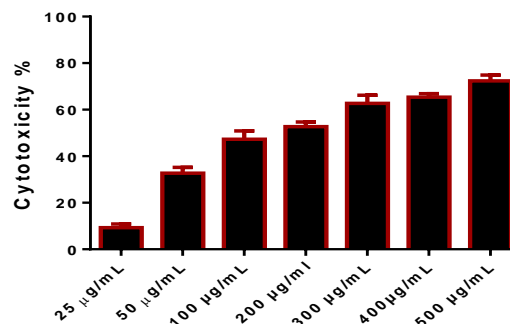


Fig. 14: Cytotoxic result of **5b** in MCF-7 cells. IC₅₀=220.19 µg/ml

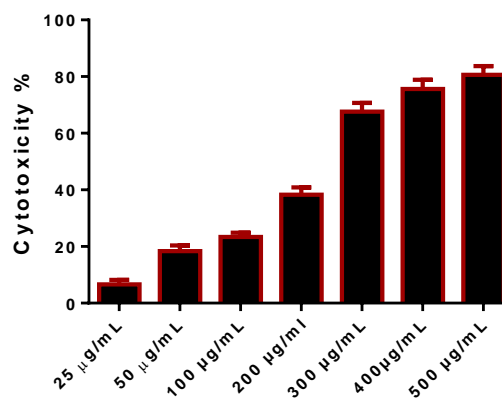


Fig. 15: Cytotoxic result of **5c** in MCF-7 cells. IC₅₀=280.12 µg/ml.

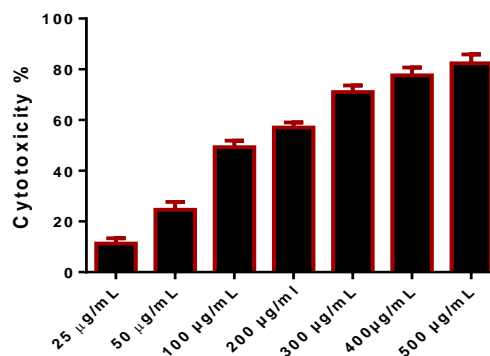


Fig. 16: Cytotoxic result of **5d** in MCF-7 cells. IC₅₀=200.07 µg/ml.

Conclusion

Four novel Prodrug maleimide derivatives were successfully prepared using ATR-FTIR and ¹HNMR techniques. All of the synthetic compounds had wider inhibition zones, which suggested strong antibacterial activity. All substances have a beneficial influence on a breast cancer cell line MCF-7. The prepared may be a viable and promising method for developing potent drugs for therapeutic use against breast tumors, according to cytotoxicity testing.

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