Synthesis, Characterization of Poly Heterocyclic Compounds, and Effect on Cancer Cell (Hep-2) In vitro.

Olfat A.Nief1* Ebtihal K. Abdullah2 Marwa N.Jasim4

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Abstract:
A synthesis series of new heterocyclic derivatives (A2–A7) (pyrrole, pyridazine, oxazine and imidazol) derived from 4-acetyl-2,5-dichloro-1-(3,5-dinitrophenyl)-1H-pyrrole-3-carboxylate(A1) have been synthesised. Synthesis of compound (A2) by the reaction of starting material (A1) with hydroxyl amine hydrochloride in the presence of pyridine. Compound (A3) was reacted with hydrazine hydrate in dry benzene to give (A3) derivative. The compound (A3) deals with sodium nitrite to give diazonium salt, and the reaction diazonium salt with ethyl acetocacetate to produce compound (A4). To a mixture of compound (A4) and hydroxyl amine with stirred to yield (A5). Compound (A6) was prepared by reaction compound (A4) with thiosemicarbazide in presence of drops of acetic acid. Synthesis of compound (A7) by reaction compound (A4) with ethyl chloroacetate. The reactions have been monitored by TLC and the synthesized compounds were characterized using spectrophotometric methods FT-IR, 1H NMR. The biological effects of the prepared compounds on the cancer cells were studied in vitro. The results indicated that these synthesized compounds (A2–A7) inhibited the cancer1 cells1 efficiently, the compound (A6) was activity inhibited on the cancer cells.

Keywords: Pyrrole, Pyridazine, Oxazine, Diazonium salt, Cancer cells.

Introduction:
Five membered ring is a branch of heterocyclic organic compounds, such as azoles that containing nitrogen atoms included at least one other heteroatom like sulfur or oxygen, and they are considered to be derived from pyrrole, furan and thiophene by replacing methine groups (-CH=) by nitrogen (-N=) atoms from the various placement (1). Pyrroles are an important class of organic compounds, that consist of a heterocyclic five membered ring (2,3). Pyrrole and its derivatives use in pharmaceutical chemistry. Pyrrolesubunit has diverse applications in therapeutically active compounds including fungicides, antibiotics, cholesterol reducing drugs, antitumor agents and any more. They are known to inhibit1 reverse transcriptase [human immunodeficiency virus1 type (HIV-1)] and cellular DNA polymerases protein kinases.

Moreover, they are also a component of polymers, indigoid dyes and of larger aromatic rings in catalytic reactions (4–10). Preparation of pyrrole is through a number of known interactions such as: Hantzsch synthesis, Knorr synthesis, Barto-Zord synthesis, Piloty-Rabinson synthesis, cycloaddition based routes and other methods (11–18). Pyridazine is a six-membered heterocyclic with low adjacent (N) atoms, Its used as an intermediate compound for the preparation of compounds containing pyrazole in structural (19,20). It is also found as a structural unit in the preparation of natural products. Pyridazines skeleton can be considered as one of the most important molecules which grant potential biological and pharmaceutical activity. Pyridazine derivatives constitutes framework of the molecule used in herbicides such as credazine, pyridafol and pyridate. It is also an important pharmacophore of top selling pharmaceutical drugs such as, Azelastine, Ameziniummetalilsulfate, Emorfazone, Cadralazine, Hydralazine, Minaprine and
Sulfamethoxypyridazine. Pyrazidine molecule and its derivatives are also known to possess a wide range of biological activities, such as anticancer, antiviral, antituberculosis, antidepressant, analgesic, antimicrobial and in platelet aggregation (21–25). The aim of the reasearch is the synthesis and characterization of organic compounds and study the effect of compounds on cancer cell line (Hep-2).

Material and Methods:

Melting points were determined on galenkamp (MFB-600), m.p apparatus and are uncorrected. FT-IR spectra of compounds were recorded on (FT-IR 8400S-spectrometer) as KBr disk). ¹H -NMR spectra (solvent DMSO-d6) were recorded on Bruker DMX-500 spectrophotometer -300MHz with in solution with TMS as internal standard. Measurements were made at the Chemistry Department, AL-AlbaitI University, Jordan.

Synthesis of ethyl 4-acetyl-2,5-dichloro-1-(3,5-dinitrophenyl)-1H-pyrrole-3-carboxylate (A1)

This is a modification of previously published synthesis (26).

Synthesis of 5,7-dichloro-6-(3,5-dinitrophenyl)-4-methylpyrrolo[3,4-d][1,2]oxazin-1(6H)-one (A2)

To a mixture of (0.001 mole, 0.3805 gm) compound A1 and (0.002 mole 0.139 gm) hydroxy amine hydrochloride, 15 mL absolute ethanol and 0.5 mL pyridine were added and the mixture was stirred for two hours at room temperature, then half of the amount of ethanol was evaporated, followed by filtration and returned crystallized from ethanol, the yellow crystals formed, filtered off (yield: 63%, m.p. 210-212 °C).

Synthesis of (Z) -5,7-dichloro-6-(3,5-dinitrophenyl)-1-hydrazono-4-methyl-1,6-dihydropyrrolo[3,4-d][1,2]oxazin (A3)

To a solution of compound A2 (0.001 mole, 0.385gm) in dry benzene (15 mL), hydrazine hydrate (6 mL) was added. The mixture was refluxed for 8 hours. The solvent was removed under vacuum and the solid product was collected and crystallized from ethanol (yield: 53%, m.p. 178-180 °C).

Synthesis of 3-((E)-((Z)-5,7-dichloro-6-(3,5-dinitrophenyl)-1H-pyrrolo[3,4-d][1,2]oxazin-1(6H)-ylidene)triaz-1-en-1-yl)pentane-2,4-dione (A4) (27).

1) In a 50ml Erlenmeyer flask (0.001, 0.399 gm) of compound (A3) was dissolved in (15ml) of water containing( 2ml ) of 6M HCl. solution is cooled to (0-5) °C in an ice bath, and a solution of (0.24gm) of sodium nitrite in 2ml of water was slowly added. Cool the Erlenmeyer flask in an ice water bath for (0 min.). The diazonium salt precipitates, keep the suspension in the ice bath so we need it in the next step of synthesis compound (A4).

2) ethyl acetoacetate (0.05 mol,6.5 gm) was added at room temperature to a stirred suspension of 1diazonium salt of compound A3 (0.01 mol) in water (20 mL) and stirring was continued for 10 min. The reaction temperature was then gradually raised to 50 °C, and after 10 minutes a clear faintly yellow solution was obtained. The solution was then treated with KOH (0.1 mol) in methanol (20 mL) and this mixture was heated to reflux and then left to crystallize. Crystals were collected by filtration, washed with acetone (yield: 72%, m.p. 203-205 °C).

Synthesis of 4-((Z)-((Z)-5,7-dichloro-6-(3,5-dinitrophenyl)pyrrolo[3,4-d][1,2]oxazin-1(6H)-ylidene)triaz-1-en-1-yl)-3-methylisoxazol-5(4H)-one (A5)

To a mixture of (3.805gm,0.01 mole) [A4] and (1.39 gm,0.02 mole) hydroxyl amine hydrochloride, 15 mL absolute ethanol and 0.5 mole pyridine were added , mixed for two hours, then cooled to room tempreture. Then, the solution was constricted by evaporating half amount of ethanol, separates by filtration and returned crystallized from ethanol, the yellow crystals formed, filtered off (yield: 51%, m.p. 243- 245 °C).
Data was analyzed on 492 nm, C C C □ C 1 C 6 (0.01 mol) and H O O 3 2 2 I I C N N (E)-2-(4-((E)-(Z))-5,7-dichloro-6-(3,5-dinitrophenyl)-4-methylpyrrolo[3,4-d][1,2]oxazin-1(6H)-ylidene(triaz-1-en-1-yl)-3-methylisoxazol-5(4H)-ylidene)hydrazine-1-carbothioamide (A6)

To a mixture of compound A5 (0.509 gm, 0.001 mole) and thiosemicarbazide (0.091 gm, 0.001 mole) in 50 mL ethanol, drops of acetic acid was added. The mixture was heated under reflux for (5-6) hours, then the mixture was cooled which resulted in solid product. The solid was filtered off, and purified by recrystallization with ethanol (98%) to give (A6) (yield: 58%, m.p. 210-212 °C).

3-Trypsin Solution: The solution was used for dismantling cell-cell.
4-Neutral red stain: The stain was used to differentiate between a living cell and a non-living one.
5-Trypan blue stain.
6-Elution Buffer.

Cell transplant and implantation conditions
In this study, Hep -2 cells type have been utilized. These cells have been grown into a single-layer form of spindle shape. They were implanted in a RPMI 1640 medium with a 10% complementary fetal calf serum, 50 mg /ml streptomycin and 1000 units/ml Penicillin (antibiotic drug against microbes). Cells line had grown into a form of one single layer under humid atomospher, temperature at 37°C and 5% CO2. Matianing pH number of the medium at a fixed value. Those experiments have been conducted on living cells at specific logarithm growth.

Cytotoxicity study
This method, previously mentioned by Vrshena (2000), has been followed. Cells suspension was prepared by adding (2) ml of Trypsin solution into (25) cm² flask of implanted cells. During suspension a cell is shown in ( 20ml ) of growth medium and then completed with 10% of fetal calf serum. The strength of the cells was calculator by the use of blue dye(Alatirepan) and should be more than 95%. After cell suspension mixed well, then was followed by transferring (200) m to 95 different deep flat plates, with pipette containing (1 x 105 cells / plate). The plate was incubated at 37°C until 60-70% after a adding (0.001 M) of prepared compounds. Plates were incubated at 37°C in an incubator, with the addition of CO2 as a complementary for 72 hour, when incubation period has completed. 50 ml/plate of neutral red stain was added. Then plate's content was removed by washing the cells three times with phosphate buffer solution. To remove the excess stain from vital cells, 100 ml of elution buffer (phosphate buffer solution and ethanol) was added. Data was analyzed on 492 nm wavelength. Inhibition rate for each compound has been determined according to the following equation:
Inhibition rate = (examination Abs 492 nm - 492 nm to control the Abs) / (492 nm to control)

Results and Discussion:
TLC test was performed on all of the prepared coumpounds, including the intermediate stages of each compound, until the reaction completed. The synthesis compounds were characterized by FTIR and 1HNMR spectroscopy.
Data analysis helped identifying the presence of functional groups in each compound, and conforming the targeted compounds' structures. The linear pathway strategy of all these synthesized compounds can be summarized in Scheme 1.

Scheme 1. Synthesis scheme of 4-acetyl-2,5-dichloro-1-(3,5-dinitrophenyl)-1H-pyrrole-3-carboxylate derivatives

**Infrared**
Compound A1 was identified by IR spectroscopy (Fig. 1): FT-IR spectrum for compound (A2) Band absorptions that is shown in the diagram was attributed to the following functional groups: band 1728 cm\(^{-1}\) (C=O) Ketone, 1735 cm\(^{-1}\) (C=O) Ester.
Treating compound A₁ with NH₂OH.HCl, afforded compound A₂ in a good yield. IR spectra (Fig. 2) showed disappearance of the absorption band (C=O) for ketone and ester, appearance of a new band at 1685 cm⁻¹, indicated cyclic formation. On the other hand, new bands appeared at (1590 cm⁻¹) attributed for (C=N)group which indicate cyclic formation.

Reacting A₂ with hydrazine afforded compound A₃. The structure of this compound was confirmed by IR spectrum (Fig. 3) which display appearance of absorption bands at (3265-3137) cm⁻¹ due to NH₂, and in the same time carbonyl group absorption band at 1685 cm⁻¹ disappeared.

Compound A₄ was prepared by reacting A₃ with ethyl acetoacetate and (NaNO₂/HCl). The reaction mixture was cooled and stirred for the required time, and the band at (3165-3137) cm⁻¹, which assigned to NH2 functional group in compound (3) disappeared. The absence of this band indicates the conversion of the NH2 group to azo group.

Compound A₅ was formed by ring closure. Using hydroxyl amine hydrochloride in a mixture of ethanol and pyridine. This reaction resulted in a formation of compound A₅. The compound was identified by IR spectra (Fig. 4). It showed one peak at 1727 cm⁻¹ which attributed for (C=O) group, associated with ring formation.
Compound A₆ was prepared by Schiff’s reaction. Compound A₅ reacted with thiosemicarbazide in ethanol, with few drops of acetic acid, which leads to the formation of the targeted compound. The IR spectrum of compound A₆ (Fig. 5) shows band absorption disappearance at 1720 cm⁻¹. However, new absorption bands appeared at 3300-3250 cm⁻¹, which was signed for (NH₂) stretching, and 3245 cm⁻¹ for (NH).

Compound A₇ was prepared by the reaction of compound A₆ with concentrated sulphuric acid. The structure of this synthesized compound was assigned on the basis of IR, ¹H NMR spectral data. The IR spectra for this compound (Fig. 6) showed the disappearance of two bands at 3300-3250 cm⁻¹ (NH₂ stretching absorption bands), and the appearance of one absorption band at 2230 cm⁻¹, which attributed for (SH) stretching. Unexpectedly, the IR spectra of this compound shows two bands associated with (NH,SH), which was attributed to the compound having two different tautomirasisim forms.

¹H NMR Spectra
The prepared compounds (A₁ to A₇) were characterized by HNMR spectroscopy. HNMR data analysis supported the structure formation compound A₁.

¹H NMR spectrum of A₁ compound (Fig. 7) shows the following characteristics: Peaks at (δ, ppm, DMSO-d⁶): 1.2-1.5 (t,2H , CH₃), 2.7 (s,3H,CH₃), 2.9-3.4 (q, 2H, CH₂) and 7.61-7.71 (m, H aromatic).
The appearance of three protons instead of the six protons showed earlier, confirms the formation of compound A₂.

In this chart, the signal at (6.4 ppm) disappeared, which indicates that (NH₂) group is no longer present. Instead, new signal shows at (3.1 ppm), the signal was assigned for methyl group (CH₃). After integration, six protons were found to be present, which means that the signal was for two methyl groups (2CH₃). This confirms the preparation of A₄ compound.
1H NMR spectrum of the compound A₇ (Fig. 11) shows significant peaks at (δ, ppm, DMSO-d6); 2.3 (s, 6H, 2CH₃), 4.7 (s, 1H, SH), 7.0-8.0 (m, H, aromatic) and 13.2 (s, 1H, NH). The new signals that attributed to the presence of CH₂ group, and the disappearance of NH₂ peaks supported the idea of tautomeric forms being present in the compound.

Figure 10 . ¹H-NMR spectrum for compound(A₆ )

Figure 11 . ¹H-NMR spectrum for compound(A₇ )

The effect of compounds (A₁-A₇) on cancer cell line (Hep-2)

One type of cancer cell line has been used to investigate how the compounds under study. Affect the cell growth in vitro. The cell line used was Hep-2 type. In this method, the number of cells is calculated under the optimal condition for cell growth. Then the prepared compounds were added to investigate their effects on cell growth (Hep-2).

At the end of the test of the prepared compounds, it was found that the value of T(cytotoxic to MT-4Cells at Cₛ₀) was 2.873 at the degree of freedom 53, and the value of the P-value was 0.023, which is less than 0.05, indicating a statistically significant difference between the compounds (Tables 1.2)

Table 1. Test One-sample test

| Rate of inhibition | d.f. | Sig. (2-tailed) | Mean of difference | 95% confidence internal of the difference |
|-------------------|------|----------------|--------------------|------------------------------------------|
| T(cytotoxic to MT-4Cells at Cₛ₀) | 2.873 | 53 | 0.023 | 2.89807 | Lower 0.7025  Upper 5.4306 |

Table 2. Inhibition values of prepared compounds

| No. Comp. | A₁ | A₂ | A₃ | A₄ | A₅ | A₆ | A₇ |
|-----------|----|----|----|----|----|----|----|
| Inhibition value | 72.94 | 67.20 | 77.24 | 73.24 | 76.51 | 81.54 | 80.19 |

Conclusion:
The present work reports a new investigation of heterocyclic systems (pyrrole, pyridazine, oxazine and imidazol) derived from 4-acetyl-2,5-dichloro-1-(3,5-dinitrophenyl)-1H-pyrrole-3-carboxylate. And the systems were prepared with the objective of
developing better antibacterial molecules. Note that when the number of heterocyclic increase in the compound its inhibitory capacity increases. The seven compounds have the highest inhibition capacity.

**Conflicts of Interest:** None.

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تخليق، تشخيص مركبات غير المتجانسة متعددة، وتأثيرها على الخلايا السرطانية (Hep-2) خارج الخليه

الله عبد نافئ 1  ابتهال قحطان عبد الله 2  هالة محمد غريب الزهاوي 3  مروه نصيف جاسم 4

1 قسم الكيمياء، كلية العلوم، الجامعة المستنصرية، بغداد، العراق
2 قسم الكيمياء، كلية العلوم، جامعة تكريت، كركوك، العراق
3 قسم الكيمياء، كلية العلوم، جامعة كركوك، كركوك، العراق
4 قسم الفيزياء، كلية التربية، الجامعة المستنصرية، بغداد، العراق

الخلاص:
تحضير سلسلة من المشتقات غير متجانسة الجديدة (A2-A7) بين بيرول، بيريديزين، أوكسازين، (اميدازول) المشتقة من (A1).

1 تحضير المركب (A2) عن طريق تفاعل مادة الأولية (A1) مع هيدروكلوريد أمين الهيدروكربون. تم تفاعل المركب (A2) مع الهيدروكربون المائي في البنزين ليعطي مشتق مشتق (A3) مع ترتيت الصوديوم لإعطاء ملح الديازونيوم، وأيضا تفاعل ملح الديازونيوم مع آلي استيسيات لينتج المركب (A4).

2 تفاعل المركب (A2) مع الهيدروكربون المائي في البنزين ليعطي مشتق (A3) مع ترتيت الصوديوم لإعطاء ملح الديازونيوم، وأيضا تفاعل ملح الديازونيوم مع آلي استيسيات لينتج المركب (A4)

3 تم تحضير المركب (A5) بواسطة تفاعل المركب (A2) مع الأمين الهيدروكربون. تم تفاعل مركب (A3) مع الهيدروكربون المائي في البنزين ليعطي مشتق (A4) مع ترتيت الصوديوم لإعطاء ملح الديازونيوم، وأيضا تفاعل ملح الديازونيوم مع آلي استيسيات لينتج المركب (A5).

4 تفاعل المركب (A1) مع الهيدروكربون المائي في البنزين ليعطي مشتق (A2) مع ترتيت الصوديوم لإعطاء ملح الديازونيوم، وأيضا تفاعل ملح الديازونيوم مع آلي استيسيات لينتج المركب (A3).

الكلمات الفئوية: بيرول، بيريدازين، أوكسازين، أملاح الديازونيوم، الخلايا السرطانية