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Design, synthesis and preliminary pharmacological evaluation of new possible non-steroidal anti-inflammatory agents having the 5-(methylsulfonyl)-1,2,4-triazole-3-amine pharmacophore

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ABSTRACT

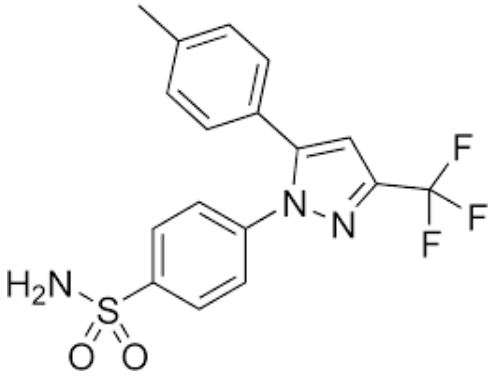
Design and synthesizenew derivatives of well-known classical NSAIDs with an increased bulkiness and expected selectivity towards the COX-2 enzyme, while being devoid of ulcerogenic effects. The target compounds were synthesized by conjugating an amino derivatives [5-(methylsulfonyl)-4H-1,2,4-triazol-3-amine] as an amide with the carboxyl group of a number of well-known NSAIDs. Synthetic procedures have been successfully developed for the generation of the target compounds (T_1-T_4). The structure of the synthesized derivatives has been characterized by elemental microanalysis (CHN), FTIR Spectroscopy, and other physicochemical properties. In vivo acute anti-inflammatory activity of the final target compounds (T_1-T_4) was evaluated in rats using the egg-white induced edema model of inflammation in a dose equivalent to (3 mg/Kg) of Diclofenac Sodium. All tested compounds produced a significant reduction in paw edema with a continued effect till the end of the experiment in respect to the effect of propylene glycol 50% v/v (control group). Moreover, target compound T_3 exhibited superior anti-inflammatory activity compared to Diclofenac Sodium at times 180-300 minutes with the same onset of action. Theincorporated 5-(methylsulfonyl)-4H-1,2,4-triazol-3-aminePharma-cophore into the final target compounds has maintained and even synergizedthe anti-inflammatory activity of the chosen classical NSAIDs and this may be due to an increased selectivity towards the COX-2 enzyme, a factor which need to be confirmed in future by assessing COX-1:COX-2 inhibitory ratios.

Keywords: Anti-inflammatory activity, paw edema, 5-(methylsulfonyl)-4H-1,2,4-triazol-3-amine, Mefenamic Acid, Indomethacin, Naproxen, and Diclofenac.

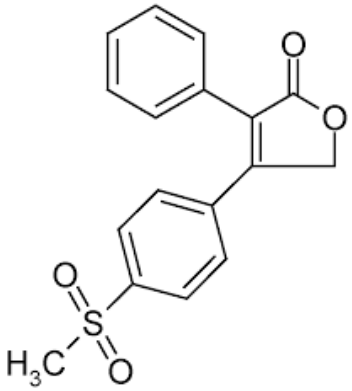
INTRODUCTION

The prostaglandins are lipid autacoids derived from arachidonic acid and are synthesized via the cyclooxygenase pathway. Two related isoforms of the cyclooxygenase enzyme have been described. Cyclooxygenase-1 (COX-1) is responsible for the physiological production of prostanoids whereas the cyclooxygenase-2 (COX-2) causes the elevated production of prostanoids that occur at the site of disease and inflammation. COX-1 is described as a "house-keeping enzyme" that regulates normal cellular processes such as gastric cytoprotection, vascular homeostasis, platelet aggregation and kidney function. COX-2 is constitutively expressed in some tissues such as the brain, kidney, and bone, and its expression at other sites is increased during states of inflammation. The two enzymes share sixty-present homology in their amino acid sequence. However the involved conformations for the substrate binding sites and catalytic regions are slightly different for example, COX-2 has a larger and more flexible substrate channel than COX-1 has, and in addition COX-2 has a larger space at the site where inhibition bind and this structural difference between COX-1 and COX-2 has permitted the development of COX-2 selective inhibiter [1]Classical non-steroidal anti-inflammatory agents (NSAIDs) inhibit both isozymes to different extents, a feature that was attributed to the corresponding differential tissue distribution of the latter and also accounts for the shared

therapeutic properties of these drugsas well as their side effects[2].Classical NSAIDs were demonstrated to induce gastric damage by a dual insult mechanism, since they are acidic in nature so the damage to the GI tract will be brought about by changing the permeability of cell membrane allowing a back diffusion of hydrogen ions, resulting in cell damage[3]; while on the other hand the nonselective inhibition of prostaglandin biosynthesis in the GI tract prevents the prostaglandin from exerting theirprotective mechanism on the gastric mucosa [4]. The differential tissue distribution of COX-1 and COX-2 has provided a rationale for the development of selective COX-2 inhibitors as non-ulcerogenic, anti-inflammatory and analgesic agents that seemed to lack the GI and hematologic liabilities[5] exhibited by the currently marketed NSAIDs [6,7]. This hypothesis has been validated in animal models and has led to the marketing of two1,2-diarylheterocycles, celecoxib (1) and rofecoxib (2) as selective COX-2 inhibitors (Figure 1) [8-13].In addition the findings that the biochemically based strategy for the facile conversion of carboxylate containing NSAIDs into esters or amides have led successfully to the production of several potent selective COX-2 inhibitors [14], but there is still an evidence to suggest that COX-2 selective inhibitors may inhibit COX-1 and induce GI irritation or ulceration with long term use or at higher doses [15,16]. Preclinical cardiovascular and renal liabilities of at least some COX-2 selective inhibitors have also been reported [17].However, there is still a need for new, selective COX-2 inhibitors with an improved safety profile.



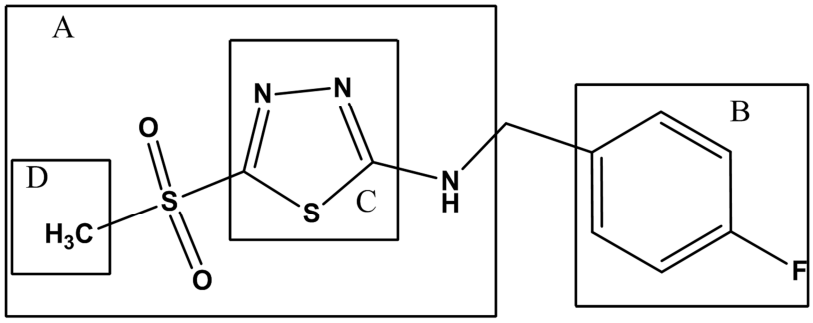
Celecoxib (1)



Rofecoxib (2)

Figure 1: The structures of celecoxib and rofecoxib

Recently, a new class of COX-2 candidates was reported where the design, synthesis and evaluation of the anti-inflammatory, analgesic, and antiplatelet properties of new 1,3,4-thiadiazole derivatives was structurally planed by exploiting the molecular hybridization approach between the diuretic drug acetazolamide and a 1,3-benzodioxole,which is a COX-2 inhibitor, previously developed. The design of the work suggested the construction of a COX-2 Pharmacophore which consist of 5-amino-1,3,4-thiadiazole-2-sulfonyl moiety and the presence of four requirements (A, B, C and D) which aid this model and are presented in Figure 2 . The *in vivo* pharmacological evaluation of these new compounds has led to the identification that the *para*-fluoro-substituted derivative (3) (Figure 2) is a new prototype which is more active than celecoxib at the same molar concentration and this could be due to selective COX-2 inhibition [18].



***N*-(4-fluorobenzyl)-5-(methylsulfonyl)-1,3,4-thiadiazol-2-amine**

(3)

Figure 2: Design concept of new 1,3,4-thiadiazole COX-2 inhibitor candidate

Therefore, the scope of this project was designed to synthesize a new structural pattern of selective COX-2 inhibitors,with possible synergistic activity and better safety margin, by the conjugationof an amine Pharmacophore namely [5-(methyl-sulfonyl)-4H-1, 2, 4-triazol-3-amine] as an amides with the carboxyl group of a number of well-known classical (NSAID).

MATERIALS AND METHODS

Materials and Equipments:
Aminoguanidine sulphate was purchased fromProvizer pharma (India), Carbon disulfide was purchased from Riedel-de Haën (Germany), Dicyclohexyl carbodiimide and Methyl iodide were purchased from Hopkin and williams Ltd (England), Hydrogen peroxide was purchased from Emirates (UAE), Propylene glycol was purchased from Avon Chem (U.K.), Tetrahydrofuran was purchased from Fluka AG (Switzerland), and Zinc dust was purchased from Merck (Germany). Diclofenac Sodium, Mefenamic acid, Indomethacin and Naproxen were donated thankfully from The State Company for Drug Industries (SDI, Samara, Iraq).

The quality of all these chemicals together with the other ones used throughout the study and obtained from standard commercial sources were of the highest purity available and used without further purification.

The melting points were determined by the open capillary method using Thomas hoover (England) and were used uncorrected. Cooling of reactions when needed was done using a Julabo chiller VC (F30) (GMBH, Germany). Infra-red spectra were recorded in KBr disc on Shimadzu FTIR 8400S spectrophotometer (Japan), at the College of Science - University of Karbala. Elemental microanalysis was performed using CHN Euro EA Elemental analyzer (Italy), at the College of Pharmacy - University of Karbala.

The progress of the reaction was monitored by ascending thin layer chromatography which was run on Kieslgel G60 F_{254} pre-coated 0.2 mm thickness Aluminum plates (E. Merck, Germany), and was used as well to check the purity of the product. The synthesized final products and their intermediates were revealed either by derivatization or reactivity toward iodine vapor or by irradiation with UV_{254} light. The chromatograms were eluted by using the following solvent systems: solvent systems **(A):** chloroform: ethyl acetate: acetic acid (7: 2.5: 0.1 v/v)[19]and solvent system **(B):** THF: ether: n-hexane (4: 4: 2 v/v)[20].

General chemical tests such as the sodium fusion or other specific suitable tests were run to check the presence or absence of certain groups and the purity of the synthesized derivatives and intermediates[21,22].

The biological evaluation of the anti-inflammatory activity of the final target compounds was performed at the Department of Pharmacology, College of Pharmacy - University of Karbala.

Experimental Section:
A. Chemical synthesis:
1. Synthesis of 5-amino-4H-1,2,4-triazole-3-thiol (Compound 3)
Compounds 3 through 5 were synthesized according to the synthetic pathway depicted in Scheme 1. Anaccurately weighed amount of aminoguanidine sulphate (1) (0.021 mol, 3.57 g) was dissolved in (15ml) of absolute ethanol contained in a 250 ml round bottom flask, and to this solution sodium carbonate (0.01 mol, 1.06 gm) and CS_2 (2) (0.063 mol, 4.8 gm) were then added respectivelywith continues stirring. The reaction mixture was refluxed for 3 hours, and after which the solvent was evaporated to dryness under vacuum.

The obtained residue was dissolved in about 7 ml of cold distilled water, and then (1 ml) of concentrated HCl was added cautiously. The white precipitate formed was filtered and washed with ice cold distilled water, and then recrystallized from hot distilled water to afford a faint white powder of compound (3).

The physical appearance, percent yield, melting point (m.p. ^{o}C) and R_f values are given in tablet 1. The FTIRspectral data(KBr) υ cm^{-1} are: 3379 and 3167 (N-H) stretching vibrations of primary amine, 3265 (N-H) stretching vibration of secondary amine,2651 (S-H) stretching vibration of thiol,1645 (N-H) bending vibration of primary amine,1539(N-H) bending vibration of secondary amines,1371 (C-N) stretching vibration of tertiary aromatic amine, and 1060 (C=S) stretching vibration that gives an evidence that compound (3) can exist in two tautomeric forms, the thiol and thione forms .

2. Synthesis of 5-(methylthio)-4H-1,2,4-triazol-3-amine (Compound 4)
Compound (3) (0.01 mol, 1.16 g) was dissolved in the minimum volume of distilled water (about 5 ml), and a sufficient volume of (85% w/v) KOH solution (5 ml) was then added under continuous stirring at room temperature

(RT). After 5-10 minutes (min.), the solution was brought to 0 °C in an ice bath and CH_3I (0.01mol, 0.623ml) was added with vigorous stirring at a rate of one drop every 2min., after which the temperature (temp.) and stirring were maintained for another 1hour (hr.). Later the solvent was evaporated to dryness under vacuum and to the obtained residue, cold absolute ethanol (about 30 ml) was added and the mixture was filtered, and from which the formed KI was obtained as the ppt on the filter paper. The original filtrate was evaporated to dryness under vacuum, and this process was repeated twice to ensure complete removal of KI from the product. The obtained product was then used without further purification to yield compound (4).

The physical appearance, percent yield, melting point (m.p. ^oC) and R_f values are given in tablet 1. The FTIR spectral data (KBr) υ cm^{-1} are:3427 and 3308 (N-H) stretching vibrations of primary amine, 3246 (N-H) stretching vibration of secondary amine, 2931 and 2847 Aliphatic (C-H) asymmetric and symmetric stretching vibrations of CH3, 1649 (N-H) bending vibration of primary amine,1504 (N-H) bending vibration of secondary amine,1429 and 1381 Aliphatic (C-H) asymmetric and symmetric bending vibrations of CH3, 1365 (C-N) stretching vibration of tertiary aromatic amine, and 650 (C-S-C) stretching vibrations of thioether.

3. Synthesis of 5-(methylsulfonyl)-4H-1,2,4-triazol-3-amine (Compound 5)

To compound (4) (0.01mol, 1.3 g) dissolved in (30ml) ethanol (95% v/v), H_2O_2 (0.02 mol, 0.68 g) was added at room temperature with continues stirring for a period of 1 hr. Then the solvent was evaporated to dryness to give compound (5), which was used without further purification.

The physical appearance, percent yield, melting point (m.p. ^oC) and R_f values are given in tablet 1. The FTIR spectral data (KBr) υ cm^{-1} are: 3427 – 3308 (N-H) stretching vibrations of primary amine,3246 (N-H) stretching vibration of secondary amine,2933 and 2850 Aliphatic (C-H) asymmetric and symmetric stretching vibrations of CH3,1645 (N-H) bending vibration of primary amine, 1502 (N-H) bending vibration of secondary amine,1373 (C-N) stretching vibration of tertiary aromatic amine,1280 and 1195 (SO2) asymmetric and symmetric stretching vibrations of sulfone.

4. The preparation of Diclofenac [2(2((2,6dichlorophenyl)amino)phenyl)-acetic acid] from its sodium salt (Compound 9a)

Diclofenac Sodium (6.28 mmol, 2g) was dissolved in the minimum volume of ethanol (99%): tetrahydrofuran (THF) (3:1) mixture (30 ml). The solution was cooled to 18 ^oC while stirring for about 10 min., and then 3.15 ml of 2N HCl solution (6.28 mmol.) was added to it, and followed by addition of excess cold water (100 ml). The precipitated Diclofenac was filtered and dried to give compound (9a) which was used in next step without further purification.

The physical appearance, percent yield, melting point (m.p. ^oC) and R_f values are given in tablet 1. The FTIR spectral data (KBr) υ cm^{-1} are: 3323 (N-H) stretching vibration of secondary amine,2969 and 2844 Aliphatic (C-H) asymmetric and symmetric stretching vibrations,2563-2900 Broad band indicate (-COOH) group,1693(C=O) stretching vibration of –COOH group, 1587 (C=C) stretching vibrations of aromatic skeleton,1508 (N-H) bending vibration of secondary amine,1454 (C-H) in-plane bending vibration of -CH2 group,1321 Aromatic (C-N) stretching vibration of secondary amine,1199, 709 Aromatic (C-H) out-of-plane bending vibration of 1,2-disubstituted benzene,1091 Aromatic (C-Cl) stretching vibration,and 833 Aromatic (C-H) out-of-plane bending vibration of 1,2,3-trisubstituted benzene.

5. General procedure for the synthesis of the acid anhydride derivatives of the used NSAIDs (Compounds 6–9)

The anhydride intermediates (6–9) were obtained when two moles of each of the carboxylic acid-containing NSAID compounds were initially dissolved in THF (30 ml), and then one mole of dicyclohexyl carbodiimide (DCC) was added to this solution. The reaction mixture was continuously stirred at room temperature for 4 hours, whereby a white precipitate of dicyclohexylurea (DCU) was formed, which then was removed by filtration. The solvent was evaporated under vacuum to yield each one of the corresponding anhydrides (6–9) that will be used in the next step without further purification (Scheme 2) [23].

The physical appearance, percent yield, melting point (m.p. ^oC) and R_f values of the intermediate compounds 6-9 are given in tablet 1, while their FTIR spectral data (KBr) υ $cm^{-1}$are listed below beside each of the synthesized intermediate:

2-((2,3-dimethylphenyl)amino)benzoic anhydride (Compound 6):3325 (N-H) stretching vibration of secondary amine,3064 Aromatic (C-H) stretching vibration,2929 and 2856 Aliphatic (C-H) asymmetric and symmetric stretching vibrations of CH3,1797 and 1734 (C=O) asymmetric and symmetric stretching vibrations of anhydride,1629, 1577 and 1508 (C=C) stretching vibrations of aromatic skeleton,1520 (N-H) bending vibration of

secondary amine,1450 Aliphatic (C-H) bending vibration of CH3,and 1020 C-(C=O)-O-(C=O)-C stretching vibration of anhydride.

2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetic anhydride (Com-pound 7):3080 Aromatic (C-H) stretching vibration,2929 and 2850 Aliphatic (C-H) asymmetric and symmetric stretching vibrations,1801 and 1739 (C=O) asymmetric and symmetric stretching vibration of anhydride,1683 (C=O) stretching vibration of carbonyl conjugated to aromatic rings,1612 and 1475 (C=C) stretching vibrations of aromatic skeleton,1430 and 1365 Aliphatic (C-H) asymmetric and symmetric bending vibrations,1228 and 1070 (C-O-C) asymmetric and symmetric stretching vibrations of aromatic alkyl ether,1064Aromatic (C-Cl) stretching vibration,and 1026C-(C=O)-O-(C=O)-C stretching vibration of anhydride.

2-(6-methoxynaphthalene-2-yl)propanoic anhydride (Compound 8):3064 Aromatic (C-H) stretching vibration,2935 and 2852 Aliphatic (C-H) asymmetric and symmetric stretching vibrations, 1807 and 1730 (C=O) asymmetric and symmetric stretching vibrations of anhydride,1602 and 1494 (C=C) stretching vibrations of aromatic skeleton,1222 and 1078 (C-O-C) asymmetric and symmetric stretching vibrations of aromatic alkyl ether,and 1031 C-(C=O)-O-(C=O)-C stretching vibration of anhydride.

2-(2-((2,6-dichlorophenyl)amino)phenyl)acetic anhydride (Compound 9):3323 (N-H) stretching vibration of secondary amine,3064 Aromatic (C-H) stretching vibration,2928 and 2852 Aliphatic (C-H) asymmetric and symmetric stretching vibrations,1803 and 1732 (C=O) asymmetric and symmetric stretching vibrations of anhydride,1614, 1577 and 1508 (C=C) stretching vibrations of aromatic skeleton,1454Aliphatic (C-H) bending vibration,1091 Aromatic (C-Cl) stretching vibration, and 1030 C-(C=O)-O-(C=O)-C stretching vibration of anhydride.

6. General procedure for the synthesis of the final target compounds T_1–T_4

A mixture of the appropriate anhydride 6–9 (0.005 mol), intermediate compound 5 (0.01 mmol, 1.62 gm), zinc dust (0.01 gm), glacial acetic acid (1.1 ml) and dioxan (30 mL) were placed in a round bottom flask equipped with a reflux condenser, and to which boiling chips were added. The reaction mixture was refluxed gently for 90 min, and later the solvent was evaporated under vacuum. The obtained residue was dissolved in ethyl acetate and then was washed with 10 ml portions of 10% w/v $NaHCO_3$ (3x), 1N HCl (3x) and finally with distilled water (3x)to remove unreacted starting materials. Later the organic layer was dried by using magnesium sulfate and followed by filtration. The filtrate was evaporated under vacuum to give the final compounds T_1–T_4. The final products were obtained as solids, and the recrystallization was carried out by dissolving the compound in ethyl acetate and followed by the addition of petroleum ether (80–100 °C) to the solution until turbidity occurs, after which it was kept in a cold place overnight. The mixture was filtered while cold and the crystalline precipitates were collected to give the appropriate final compounds T_1–T_4(Scheme3).

The physical appearance, percent yield, melting point (m.p. ^oC) and R_f values of the target final compounds (T_1-T_4) are given in tablet 1, while their FTIR spectral data (KBr) υ $cm^{-1}$and the elemental microanalysis (CHN Analysis) are listed below beside each of the synthesized derivative:

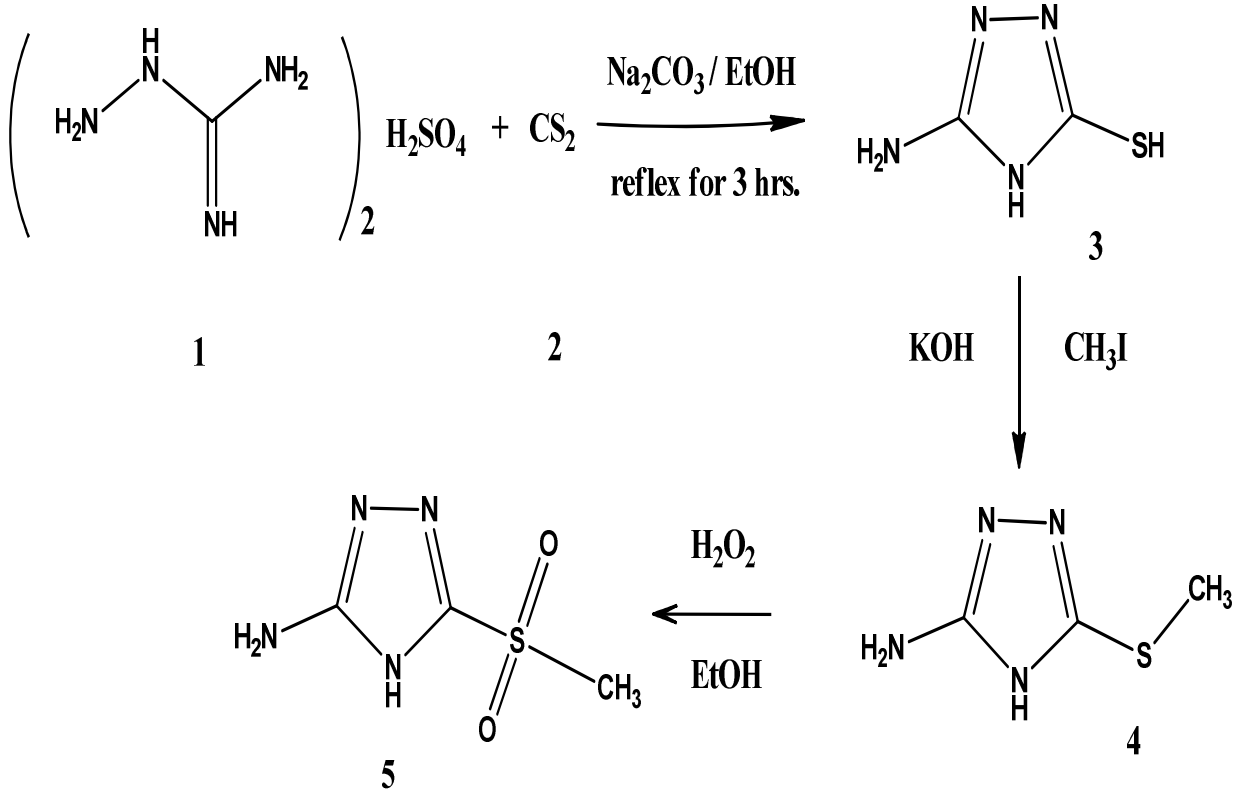
2-((2,3-dimethylphenyl)amino)-N-(5-(methylsulfonyl)-4H-1,2,4-triazol-3-yl)benz-amide (Compound T_1): The FTIR spectral data (KBr) υ cm^{-1} are: 3346 (N-H) stretching vibration of secondary amide,3311 (N-H) stretching vibration secondary amine,3014 Aromatic (C-H) stretching vibration,2918 and 2860 Aliphatic (C-H) asymmetric and symmetric stretching vibrations,1651 (C=O) stretching vibration of secondary amide (amide I),1575, 1506 and 1442(C=C) stretching vibrations of aromatic skeleton which overlap with the (N-H) bending vibration of secondary amide (amide II),1329 and 1159 (SO2) asymmetric and symmetric stretching vibration of sulfone,and 1251 (C-N) stretching vibration of tertiary aromatic amine; while theCHN Analysis calculatedfor $C_{18}H_{19}N_5O_3S$ (Mol. Wt. 385):C,56.09;H,4.97;N, 18.17.And the observed: C,56.182;H,4.851;N,18.686.

2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(5-(methylsulfonyl)-4H-1,2,4-triazol-3-yl)acetamide (Compound T_2): The FTIR spectral data (KBr) υ cm^{-1} are: 3381 (N-H) stretching vibration of secondary amide,3292 (N-H) stretching vibration secondary amine,3088Aromatic (C-H) stretching vibration,2928 and 2847Aliphatic (C-H) asymmetric and symmetric stretching vibrations,1685 (C=O) stretching vibration of secondary amide (amide I),1629 (C=O) stretching vibration of tertiary amide,1595 and 1477(C=C) stretching vibrations of aromatic skeleton which overlap with the (N-H) bending vibration of secondary amide (amide II),1390 Aliphatic (C-H) bending vibration of CH3 group, 1313 and 1147 (SO2) asymmetric and symmetric stretching vibrations of sulfone,1271 and 1041 (C-O-C) asymmetric and symmetric stretching vibrations of aryl alkyl ethers, and1078Aromatic (C-Cl) stretching vibration; while the CHN Analysis calculated for $C_{22}H_{20}ClN_5O_5S$ (Mol. Wt. 501):C,52.64;H,4.02;N,13.95. And the observed: C, 52.051;H, 3.876;N, 14.042.

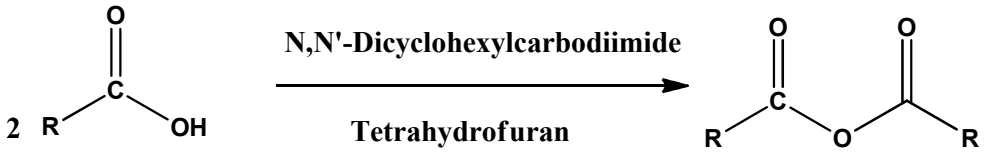
2-(6-methoxynaphthalen-2-yl)-N-(5-(methylsulfonyl)-4H-1,2,4-triazol-3-yl)propan-amide (Compound T3): The FTIR spectral data (KBr) υ cm^{-1} are: 3416 (N-H) stretching vibration of secondary amide,3282 (N-H) stretching vibration secondary amine,3057 Aromatic (C-H) stretching vibration,2964 and 2841 Aliphatic (C-H) asymmetric and symmetric stretching vibrations,1631 (C=O) stretching vibration of secondary amide (amide I),1606 and 1510 (C=C) stretching vibrations of aromatic skeleton which overlap with the (N-H) bending vibration of secondary amide (amide II),1458 and 1388 Aliphatic (C-H) asymmetric and symmetric bending vibration of CH3 group,1354 and 1163 (SO2) asymmetric and symmetric stretching vibrations of sulfone,and 1269 and 1062 (C-O-C) asymmetric and symmetric stretching vibrations of aryl alkyl ethers;while the CHN Analysis calculated for $C_{17}H_{18}N_4O_4S$ (Mol. Wt.374):C, 54.53;H, 4.85; N, 14.96. And the observed: C, 55.940;H, 4.892;N, 15.016.

2-(2-((2,6-dichlorophenyl)amino)phenyl)-N-(5-(methylsulfonyl)-4H-1,2,4-triazol-3-yl)acetamide (Compound T4): The FTIR spectral data (KBr) υ cm^{-1} are: 3497 (N-H) stretching vibration of secondary amide ,3323 (N-H) stretching vibration of secondary amine ,3072 Aromatic (C-H) stretching vibration ,2922 and 2856 Aliphatic (C-H) asymmetric and symmetric stretching vibrations ,1695 (C=O) stretching vibration of secondary amide(amide I) ,1620, 1577 and 1504 (C=C) stretching vibrations of aromatic skeleton which overlap with the (N-H) bending vibration of secondary amide (amide II) ,1450 and 1373 Aliphatic (C-H) bending vibration of CH2 and CH3 groups ,1309 and 1159 (SO2) asymmetric and symmetric stretching vibrations of sulfone ,and 1093 Aromatic (C-Cl) stretching vibration; while the CHN Analysis calculated for $C_{17}H_{15}Cl_2N_5O_3S$(Mol. Wt. 440):C, 46.37;H, 3.43;N, 15.91; And the observed: C, 46.621;H, 3.420;N, 16.009.

The general routes illustrated in Schemes 1,2 and 3 were followed to synthesize all the intermediate and final target compounds described earlier. Scheme 1 has shown that the*5-(methylsulfonyl)-4H-1,2,4-triazol-3-amine* (Compound 5) was synthesized starting from Aminoguanidine sulphate.

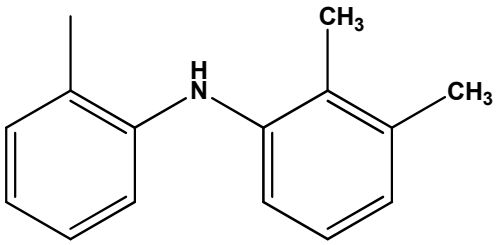


Scheme 1: The synthesis of intermediates compounds (3, 4 and 5)

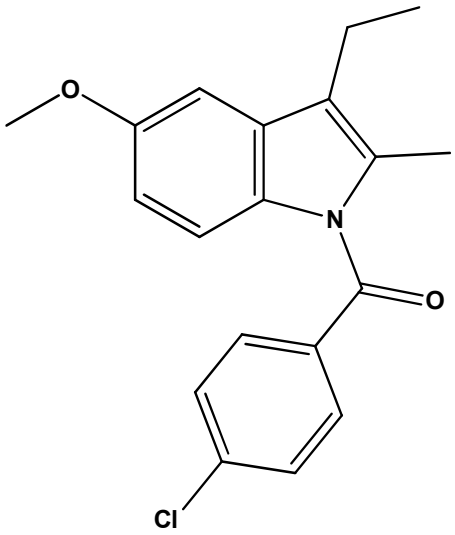


NSAIDs **compounds 6-9**

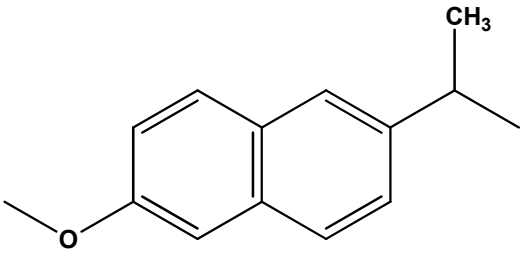
Where R =



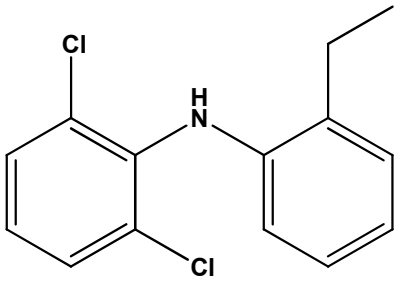
in compound 6



in compound 7

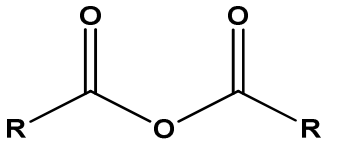


in compound 8



in compound 9

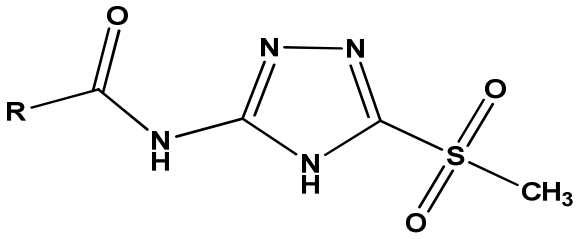
Scheme 2: The synthesis of the NSAIDs anhydride intermediate compounds (6, 7, 8 and 9)



Compounds 6-9

\+ intermediate 5

Glacial acetic acid / Zn dust / dioxane reflux for 90 min.



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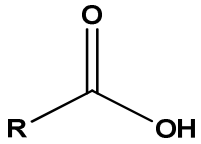
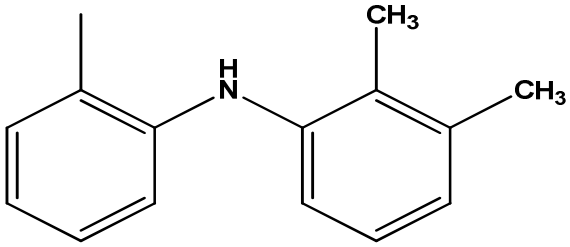
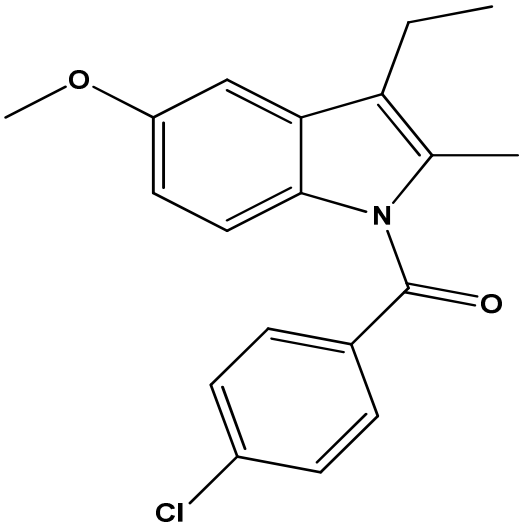


Target compounds T_1-T_4

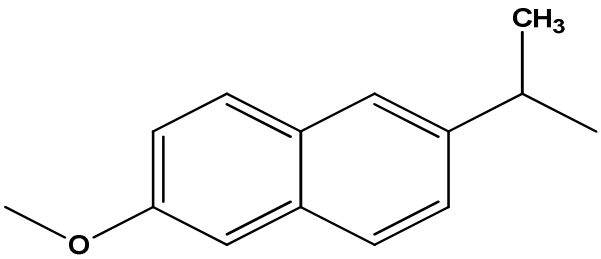
Where R =



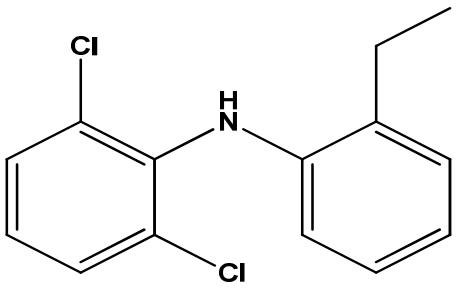
compound T_1



compound T_2



compound T_3



compound T_4

Scheme 3: The synthesis of final target compounds (T_1, T_2, T_3 and T_4)

Table 1: The percent yield, physical appearance, uncorrected Melting points, and R_f values of the synthesized intermediates and final compounds (T_1-T_4)

Com-pound	*Chemical name*	*Molecular formula*	*Physical appearance*	*% yield*	*Melting point°C*	*R_f value*
3	5-amino-4H-1,2,4-triazole-3-thiol	$C_2H_4N_4S$	White powder	70	314-316[1]	A = 0.12 B = 0.66
4	5-(methylthio)-4H-1,2,4-triazol-3-amine	$C_3H_6N_4S$	Yellow powder	33	130-133[2]	A = 0.28 B = 0.54
5	5-(methylsulfonyl)-4H-1,2,4-triazol-3-amine	$C_3H_8N_4O_2S$	Brownish yellow powder	85	>250	A = 0.2 B = 0.42
6	2-((2,3-dimethyl-phenyl)amino)benzoic anhydride	$C_{30}H_{28}N_2O_3$	Yellow powder	75	158-160	A = 0.76 B = 0.50
7	2-(1-(4-chloro-benzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetic anhydride	$C_{38}H_{30}Cl_2N_2O_7$	Pale yellow powder	63	149-151	A = 0.8 B = 0.60
8	2-(6-methoxy-naphthalene-2-yl)-propanoic anhydride	$C_{28}H_{26}O_5$	Off white powder	65	130-132	A = 0.68 B = 0.56
9	2-(2-((2,6-dichloro-phenyl)amino)-phenyl)acetic anhydride	$C_{28}H_{20}Cl_4N_2O_3$	Off white powder	60	106-108	A = 0.63 B = 0.55
9a	2-[2-(2,6-dichlorophenyl-amino)phenyl]acetic acid	$C_{14}H_{11}Cl_2NO_2$	White powder	90	160-162	A = 0.75 B = 0.46
T_1	2-((2,3-dimethyl-phenyl)amino)-N-(5-(methylsulfonyl)-4H-1,2,4-triazol-3-yl)benzamide	$C_{18}H_{19}N_5O_3S$	Yellow powder	60	224-226	A = 0.62 B = 0.46
T_2	2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(5-(methylsulfonyl)-4H-1,2,4-triazol-3-yl)-acetamide	$C_{22}H_{20}ClN_5O_5S$	Pale yellow powder	35	108-110	A = 0.6 B = 0.44
T_3	2-(6-methoxy-naphthalen-2-yl)-N-(5-(methylsulfonyl)-4H-1,2,4-triazol-3-yl)propanamide	$C_{17}H_{18}N_4O_4S$	Yellowish brown powder	37.5	118-120	A = 0.64 B = 0.50
T_4	2-(2-((2,6-dichloro-phenyl)amino)phenyl)-N-(5-(methyl-sulfonyl)-4H-1,2,4-triazol-3-yl)acetamide	$C_{17}H_{15}Cl_2N_5O_3S$	Maroon powder	33.5	127-129	A = 0.88 B = 0.55

1 = Compound 3 melts between 314-316 °C and it was reported to melt above 300 °C[24]
2 = compound 4 was also reported to melt between 130-133 °C[25]

B. Evaluation of the anti-inflammatory activity of the synthesized target compounds T_1-T_4:

The *in vivo* acute anti-inflammatory activity of the synthesized target compounds (T_1, T_2, T_3 and T_4) were evaluated using the egg-white induced paw edema method [26]. The decrease in paw thickness constitutes the basis of screening the anti-infla-mmatory activity of the newly synthesized compounds.

1. The method used:

Albino rats of either sex weighing (200 ± 10 g) were supplied by the animal house of the College of Pharmacy, University of Kerbala and were housed in the same location under standardized conditions. Animals were fed commercial chaw and had free access to water ad *libitum*. Animals were divided into six groups (each group consist of 6 rats) as follow:

Group A: six rats served as control, and treated with the vehicle (propylene glycol 50% v/v).

Group B: six rats treated with Diclofenac sodium as reference substance in a dose of 3mg/kg[27,28] dissolved in propylene glycol 50% (v/v).

Group C-F: six rats / group treated with the tested compounds (T_1, T_2, T_3 and T_4) in doses equivalent to 3 mg/kg of Diclofenac sodium and dissolved in propylene glycol 50% (v/v).

2. Dose determination:

Diclofenac sodium was used as the reference substance. The reference and tested compounds were administered by the intra-peritoneal route (i.p.). The dose of the newly synthesized compounds depends on their molecular weight and on the dose of the reference compound (Diclofenac sodium). Since the dose of Diclofenac sodium was 3mg/kg, so the doses of synthesized target compounds was calculated using the following equation:

$$\frac{\textbf{Dose of reference compound}}{\textbf{reference molecular weight}} = \frac{\textbf{Dose of tested compound}}{\textbf{tested compound molecular weight}}$$

Table 2 lists the doses calculated for the tested compounds used in the study.

Table 2: The calculated doses of the tested target compounds used in the *in vivo* anti-inflammatory study

Compound	*Diclofenac sodium*	*T1*	*T2*	*T3*	*T4*
Mol. Wt.	318	385.44	501.94	374.41	440.3
Dose mg/kg	3	3.636	4.735	3.532	4.153

3. The pharmacological evaluation procedure:
The anti-inflammatory activity of the tested target compounds was studied using the egg-white induced edema model[26]. Acute inflammation was produced by a subcutaneous injection of 0.05ml of undiluted egg-white into the planter side of the left hind paw of the rats, thirty minutes after the i.p. administration of the drugs or their vehicle (control).

The paw thickness was measured by using a vernea at seven time intervals (0, 30, 60, 120, 180, 240, and 300 minutes) after the drugs or their vehicle (control) administration i.p., which was considered as time zero.

4. The statistical Analysis:
The data are expressed as the standard error of the mean (± SEM) and the results were analyzed for their statistical significance using the student t-test (Two Sample Assuming Equal Variances) for comparison between the mean values. While comparisons between different groups were made using ANOVA (Two factors without Replication). Probability (P) value of less than 0.05 was considered as significant.

RESULTS AND DISCUSSION

The present study was designedto synthesize new derivatives of well-known classical NSAIDs with an increased bulkiness and expected selectivity towards the COX-2 enzyme, while being safer and devoid of ulcerogenic effects.The scope of the work was the synthesize of the target compounds by conjugating an amino derivatives [namely 5-(methylsulfonyl)-4H-1,2,4-triazol-3-amine] as an amide with the carboxyl group of a number of well-known NSAIDs. Synthetic procedures have been successfully developed for the generation of the target compounds (T_1-T_4) using simple methodology and with an excellent isolated yields.

The synthesis of the of the core unit [5-(methylsulfonyl)-4H-1,2,4-triazol-3-amine] started with that of 5-amino-4H-1,2,4-triazole-3-thiol intermediate (compound 3) which was obtained by condensing carbon disulfide with aminoguandine sulphate under basic condition. Then, the S-methylation of compound 3 it furnished compound 4, which was further oxidized by H_2O_2 to afford the core structure (compound 5).

The structures of the synthesized compounds were characterized and confirmed by using FTIR, elemental microanalysis (CHN), and other physicochemical parameters presented in Tables 1.

The FTIR spectra of these compounds have revealed that compound 4 showed the disappearance of the characteristic (S-H) stretching vibration of the thiol group at 2651 cm^{-1} and the appearance of the characteristic bands of the aliphatic (C-H) asymmetric and symmetric stretching vibrations of the CH_3 group at 2931 and 2847 cm^{-1} in the IR spectrum of the compound, together with the corresponding asymmetric and symmetric bending vibrations of this group at 1429 and 1389 cm^{-1}, together with the appearance of the characteristic (C-S-C) asymmetric and symmetric stretching vibrations of thioether at 1278 and 1093 cm^{-1}. On the other hand, the FTIR spectrum of compound 5 showed the appearance of the characteristic (S=O) stretching vibrations of sulfone group at 1280 and 1195 cm^{-1} and it was noticed that this band has overlapped the (C-N) stretching vibration of tertiary aromatic amine as well as the aliphatic (C-H) bending vibration of the CH_3 group.

Compounds 6, 7, 8, and 9 showed the characteristic (C=O) asymmetric and symmetric stretching vibrations of the formed anhydrides around 1800 and 1730 cm^{-1} and the band at 1025 cm^{-1} characteristic of C-(C=O)-O-(C=O)-C stretching vibration of anhydride.

The target compounds T_1-T_4 showed the disappearance of the characteristic bands that corresponds to the anhydrides and the appearance of those between 3497- 3346 cm^{-1} that corresponds to the (N-H) stretching vibration of secondary amides, together with that between 1631-1695 cm^{-1} that corresponds to the (C=O) stretching vibration of secondary amide (amide I) and finally the appearance of the characteristic bands that corresponds for the (SO_2) asymmetric and symmetric stretching vibrations of sulfone between 1309 – 1354 and 1147 – 1159 cm^{-1}.

Elemental microanalyses were performed for the target compounds (T_1, T_2, T_3 and T_4) and the results revealed good agreement with calculated percentages. The percent deviation of the observed/calculated values was found to be within the limits of accurate analysis.

Pharmacological studies:
The following data are concerned with the results of the preliminary pharmacological evaluation of the target compounds (T_1-T_4) as anti-inflammatory agents by studying the acute inflammation using the paw-edema model

produced following the subcutaneous injection of undiluted egg white into the intra-planter side of the left hind paw of the rat.

1. In Vivo pharmacological evaluation of the anti-inflammatory activity of the target compounds: The rat paw edema model:

The most widely used preliminary test to screen new anti-inflammatory agents is based on the ability of a compound to reduce local edema induced in the rat paw following injection of an irritant [29]. The pathophysiological mechanism of induced edema depends on the participation of kinins and polymorphonuclear leukocytes with their pro-inflammatory factors including prostaglandins (PGs) [30].

The onset of symptoms of inflammation and the enhanced release of PG production usually corresponds with COX-2 expression, while COX-1 appears unaffected by the inflammatory process. The direct role of PGs in the rat egg-white induced paw edema model was demonstrated by using an anti-PGE2 antibody to inhibit paw edema as effectively as Diclofenac[31]. The observation that COX-2 but not COX-1 expression is induced in animal model of acute and chronic inflammation, and that PG biosynthesis and symptoms of inflammation parallel to the COX-2 induction, were strongly support a predominant role for COX-2 in the inflammatory process[32]. When egg-white is injected into the paw of rats, a substantial induction of COX-2 is observed at 2 hours coinciding with enhanced PGs and local edema [33].

Many irritant agents have been used in the paw-edema method like brewer's yeast, formaldehyde, dextran, egg-white and carrageenan solution. The effect can be measured in several ways, where usually, the volume of the injected paw is measured before and after application of the irritant and the paw volume of the treated animals is compared to their control [34]. Many methods have been described of how to measure the paw volume by simple and less accurate methods or by more sophisticated electronically devised methods. One of these methods involves the marking of the paw with ink at the level of the lateral malleolus and then immersed in mercury contained in a cylinder fluid on a sensitive balance up to this mark and from the weight difference, the paw volume was calculated. Another method measures the paw volume plethysmographically immediately after injection, and again at all-time intervals of the test. Various devices have been developed for pleothysmograph the paw [35,36]. Webb reported a sensitive method of measuring mouse paw volume by a using a microcomputer [37].

2. Advantages of the paw edema method:
This *in vivo* method for evaluating anti-inflammatory agents has many advantages over the other methods including:

a) Rapid evaluation in which the inflammation is measured immediately and during short time course, i.e. no need to stay overnight or waiting for several days.
b) The paw is very sensitive for inflammatory substances.
c) Vernea has been used for measuring paw-volume which is a simple and more practically valid tool than others in which the change in the volume need a very sensitive capillary tube or microcomputer to be used which may not be available.
d) The method is a low cost effective because it does not involve anesthetic procedures or expensive chemical agents or dyes and in addition the rat will be conscious and alive after the end of the experiment, which reflects the human nature of the method.
e) The experimental animals used are rats, which are sensitive for induction of inflammation and respond well to the anti-inflammatory agents. In addition they are available and easily handled.
f) The difference in measurements at the various time intervals gives some indications for the onset and duration of the anti-inflammatory effect of the tested compounds.

3. Evaluation of the anti-inflammatory activity of the target Compounds:
The anti-inflammatory activity of the tested target compounds have been evaluated in comparison with their vehicle (control group) and a reference drug and presented as follows:

Effect of reference anti-inflammatory drug (Diclofenac sodium) on the paw edema:
To assess the validity of the method (paw edema) used for the evaluation of newly synthesized target compounds as an anti-inflammatory compounds, Diclofenac sodium was used as reference compound of known profile of anti-inflammatory activity. Table 3 shows that the intra-planter injection of 0.05ml egg-white at time 30 minute into the left hind paw produced significant increase in paw thickness in all animals designated as control, and Diclofenac sodium groups with respect to their baseline readings (zero time) (which is (4.5 ± 0.11 mm) for the control group and (4.49 ± 0.06 mm) for the reference group) ($P<0.05$), and furthermore, no significant difference in induced paw edema was observed among these groups.

In the control group, the paw edema was shown to be continually elevated reaching maximum (6.98 ± 0.09 mm) after 30 minutes of induction (60 minutes after the i.p. injection of the vehicle). For this reason, this time interval is used for the comparative analysis of the anti-inflammatory effect of the reference drugs and of the tested target compounds. However; paw thickness was reduced back to lower value (5.98 ± 0.09 mm) after 300 minutes (i.e. the end of experiment) as shown in figure 3. While the paw edema (at time 60 minutes) in animals previously treated with Diclofenac sodium (3mg/kg, i.p.) reached (6.65 ± 0.12mm) after 30 minutes of induction which is significantly lower when compared to that in control group ($P<0.05$), and reduced back to (5.5 ± 0.09 mm) after 300 minutes, a value found to be significantly lower in comparison with the value at time of induction ($P<0.05$) as shown in figure 3.

The differences in paw thickness readings between the control and Diclofenac sodium groups indicates that the method used in this study (paw edema) is a valid method and can effectively be used for the assessment of the anti-inflammatory effect of the newly synthesized compounds.

Table 3: The effect of Diclofenac sodium (reference compound) and propylene glycol on egg-white induced paw edema in rats

	Treated groups		
	Time (min)	**Control (n = 6)**	**Diclofenac (n = 6)**
Paw thickness (mm)	0	4.50 ± 0.11	4.49 ± 0.06
	30	6.57 ± 0.08	6.47 ± 0.11
	60	6.98 ± 0.09	6.65 ± 0.12*
	120	6.88 ± 0.10	6.47 ± 0.14*
	180	6.78 ± 0.04	6.00 ± 0.10*
	240	6.45± 0.05	5.68 ± 0.05 *
	300	5.98 ± 0.09	5.50 ± 0.09 *

Where:
The data are expressed in mm paw thickness as the mean, i. e. ± standard error of mean (SEM).
n= number of animals.
Time (0) is the time of i.p. injection of Diclofenac sodium and propylene glycol.
Time (30) is the time of injection of egg-white (induction of paw edema).
* Significantly different compared to control ($p<0.05$).

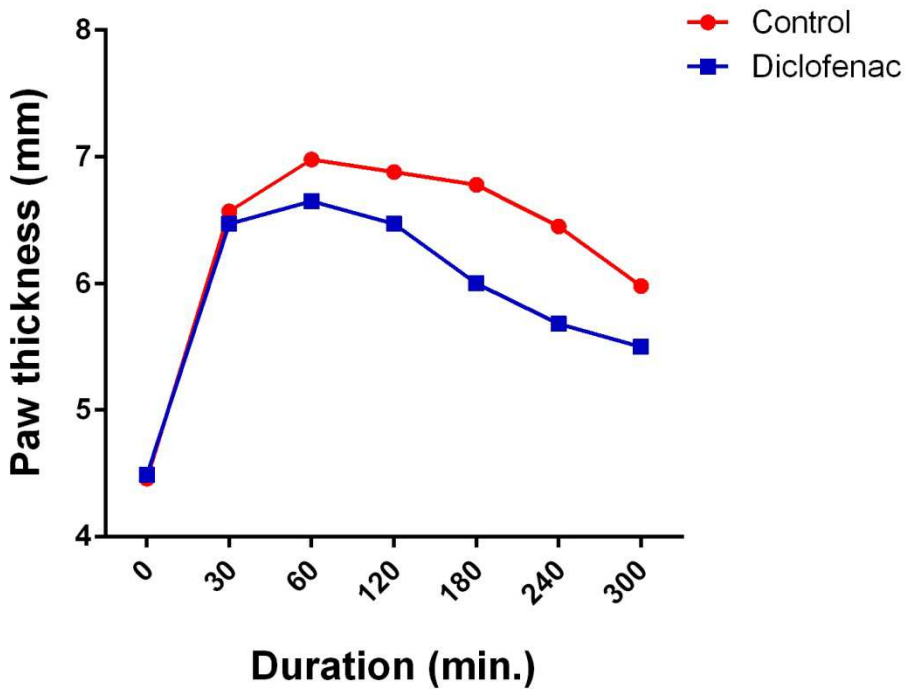


Figure 3: Effect of Diclofenac sodium (reference), and propylene glycol (control) on egg-white induced paw edema in rats
Results are expressed as the mean ±SEM (n = 6 for each group).
Time (30) is the time of egg-white injection.

Comparison the effect of tested target compounds (T_1, T_2, T_3 and T_4) with the control group:

Table (4) showed the effect of compounds (T_1,T_2,T_3 and T_4) on paw thickness after intra-plantar injection of 0.05ml egg-white. Paw edema in animals treated with compound T_1 (3.636mg/kg, i.p.) reached (6.47±0.12) after 30 minutes of induction which is significantly lower in comparison to that in control group (P<0.05) and reduced back to (5.13±0.06mm) after 300 minutes, which is significantly lower with respect to that in control group (P<0.05) as shown in figures 5 and 6. On the other hand, animals treated with compound T_2 (4.735mg/kg, i.p.) exhibited (6.63±0.07mm) elevation in paw thickness after 30 minutes of induction, a value that is significantly lower than that in control group (P<0.05). Paw thickness was decreased to (5.45±0.07mm) at the end of experiment, a value found significantly lower than that in control group (P<0.05) as shown in figures 5 and 6. Animals treated with compound T_3 (3.532mg/kg, i.p.) exhibited (6.76±0.13 mm) elevation in paw thickness after 30 minutes of induction, a value that is significantly lower than in control group (P<0.05). Paw thickness was decreased to (4.75±0.06mm) at the end of experiment, a value found significantly lower than that in control group (P<0.05) as shown in figures 3.15 and 3.16. While, animals treated with compound T_4 (4.153mg /kg, i.p.) exhibited (6.75±0.09) elevation in paw thickness after 30 minutes of induction, a value that is significantly lower than the in control group (P<0.05). Paw thickness was decreased to (4.85±0.09mm) at the end of experiment, a value found significantly lower than that in control group (P<0.05) as shown in figure 4.

Table 4: Effect of compounds T_1, T_2, T_3,$T_4$and Diclofenac sodium on egg-white induced paw edema in rats

		Treated groups				
	Time (min)	**Diclofenac sodium (n = 6)**	**Compound T_1 (n = 6)**	**Compound T_2 (n = 6)**	**Compound T_3 (n = 6)**	**Compound T_4 (n = 6)**
Paw thickness (mm)	0	4.49±0.06	4.45±0.12	4.50±0.13	4.47±0.06	4.52±0.11
	30	6.47±0.11	6.23±0.09	6.46±0.08	6.53±0.08	6.54±0.07
	60	6.65±0.12[a]	6.47±0.12[b]	6.63±0.07[a]	6.76±0.13[a]	6.75±0.09[a]
	120	6.47±0.14[a]	6.23±0.05[a]	6.14±0.08[a]	6.44±0.09[a]	5.88±0.12[b]
	180	6.00±0.10[a]	5.63±0.03[a]	5.94±0.05[a]	5.60±0.17[b]	5.58±0.05[b]
	240	5.68±0.05[a]	5.18±0.04[b]	5.67±0.14[a]	5.02±0.08[b]	5.18±0.05[b]
	300	5.50±0.09[a]	5.13±0.06[a]	5.45±0.07[a]	4.75±0.06[b]	4.85±0.0[b]

Where: The data are expressed in mm paw thickness as mean ±standard error of mean (SEM).
n= number of animals.
Time (0) is the time of i.p. injection of tested compounds, Diclofenac sodium.
Time (30) is the time of injection of egg-white (induction of paw edema).
Non-identical superscripts (a and b) among different groups are considered significantly different (p<0.05).

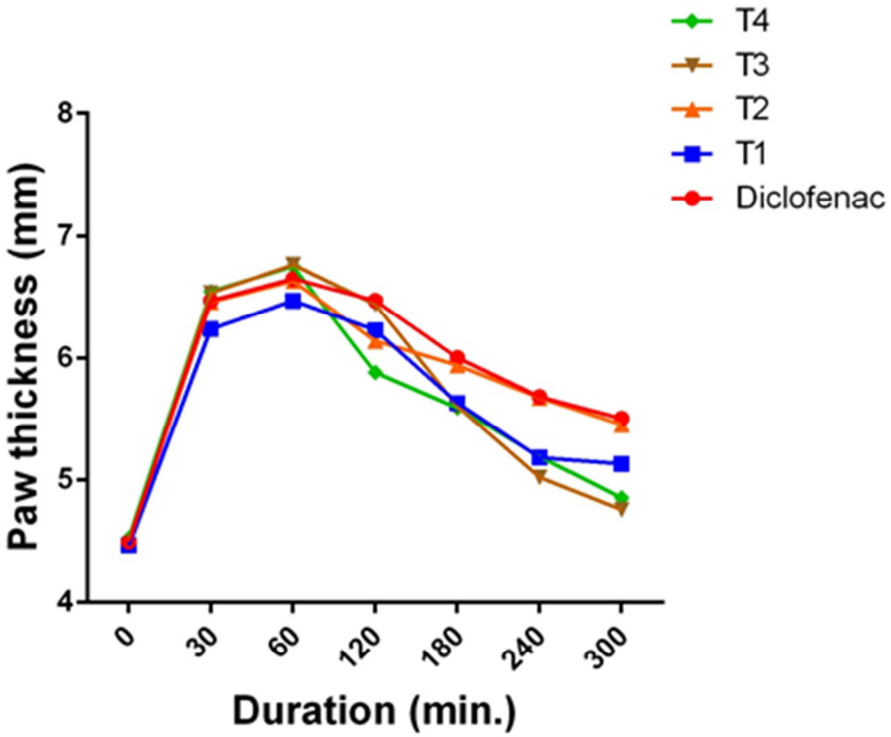


Figure 4: Effect of Diclofenac sodium, compounds T_1, T_2, T_3 and T_4 on egg-white induced paw edema in rats
Results are expressed as mean ±standard error of mean (n=6 for each group).
Time (30) is the time of egg-white injection

Comparative Analysis:
Multi-way comparison between the reference drug and tested target compounds revealed the following:

a) All tested compounds were effectively limited the increase in paw edema and their effect started 30 minutes after induction and continued till the end of the experiment as shown in figure 3.15.

b) The effect of compound T_3 and T_4 was significantly higher than that of Diclofenac sodium, at the interval time 180-300, since the original NSAIDs used or included were more potent than others and this effect was transmitted to the new derivative target compound.

c) Compound T_2 expressed a comparable effect to that of Diclofenac sodium at the interval time 180-300 minute.

d) Compound T_1 showed higher effect than Diclofenac sodium and less thancompound T_3 and T_4 at all periods of the experiment.

CONCLUSION

1. The designed compounds have been synthesized successfully and their structures were confirmed.
2. *In vivo* anti-inflammatory study of the target compounds showed that the incorporation of the 5-(methylsulfonyl)-4H-1,2,4-triazol-3-amine into the well-known anti-inflammatory drugs (Mefenamic acid, Indomethacin, Naproxen and Diclofenac) potentially increased their anti-inflammatory activity.
3. The higher efficacy and the longer duration of action of the methylsulfonyl derivatives may be attributed to their lower acidity and may have an excellent lipophilic-hydrophilic balance that will permit them partitioning through the lipid membrane of the physiological system and cause them faster reach to the site of action.
4. In vivo anti-inflammatory study for the compounds (T_3 and T_4) showed that the amidation of Naproxen and that of Diclofenac with 5-(methylsulfonyl)-1, 2, 4- triazole -3-amine Pharmacophore increased their anti-inflammatory activity.
5. Amidation of Indomethacin with 5-(methylsulfonyl)-1, 2, 4- triazole -3-amine Pharmacophore maintain its anti-inflammatory activity.
6. Amidation of Mefenamic acid with 5-(methylsulfonyl)-1, 2, 4- triazole -3-amine Pharmacophore increase its anti-inflammatory activity but less than the compounds (T_3 and T_4).

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