A Convenient Synthesis, Reactions and Biological Activity of Some New 6H-Pyrazolo[4’,3’:4,5]thieno[3,2-d][1,2,3]triazine Compounds as Antibacterial, Anti-Fungal and Anti-Inflammatory Agents

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We describe here the design and synthesis of novel pyrazolothienotriazine compounds based on diazotization followed by cycloaddition reactions of 4-amino-3-methyl-1-phenyl-1H-thieno[2,3-c]pyrazol-5-carbonitrile with sodium nitrite in the presence of concentrated HCl in acetic acid. The produced chloropyrazolothienotriazine underwent nucleophilic substitution reactions with various primary and secondary amines including sulfa drugs to afford the N-substituted aminopyrazolothienotriazines. Hydrazinolysis of the chlorotriazine with hydrazine hydrate afforded the hydrazotriazine, which was used as a versatile precursor for synthesis of other compounds. The chemical structures of the newly synthesized compounds were confirmed on the basis of elemental and spectral analyses containing Fourier transform infrared spectroscopy (FTIR), 1H and 13C nuclear magnetic resonance (NMR) and mass spectrometry. Some of the synthesized compounds showed high antibacterial and anti-fungal activities. Also, most of the tested compounds exhibited high anti-inflammatory activity compared with indomethacin using carrageenan induced rat paw edema assay.

Keywords: pyrazole, thienopyrazole, pyrazolothienotriazine, synthesis, antimicrobial activity, anti-inflammatory activity

Introduction

Pyrazoles and condensed pyrazoles are very important class of heterocyclic compounds which were considered as important scaffolds in medicinal chemistry due to their wide range of pharmacological activities; the most biological activities are anti-inflammatory,1-5 antimicrobial,6,7 antioxidant,8 anticancer,9,10 fungical,10 and antiviral activities.10,12,13 Some thienopyrazoles are used for inhibiting PDE 7 (phosphodiesterase 7) selectively, which is responsible for allergy, immunological and inflammatory diseases.14 Bindi et al.15 reported a series of thienopyrazoles to demonstrate their activities as a potent inhibitor for aurora kinase. Some members of this class of compounds have also been investigated for their local anesthetic, antiarrhythmic,16 herbicidal,17 molluscicidal properties,18 and for antiviral19 and immunosuppressant activities.20

Structurally simple 5-amino-1-tert-butyl pyrazole-4-carboxamide A was found to inhibit p56 Lck16 (Figure 1). 5-Amino-1-(4-methylphenyl)pyrazole B has been tested as an NPY5 antagonist.17 5-Amino-4-benzoyl-3-methylthio-1-(2,4,6-trichlorophenyl)pyrazole C has been reported as a potent corticotrophin-releasing factor-1 (CRF-1) receptor antagonist.18 5-Amino-1-(2,6-dichloro-4-(trifluoromethyl)phenyl)-4-(3-methoxyphenyl)-3-methylthio-pyrazole D has been described as a potent GABA (gamma-aminobutyric acid) inhibitor with selectivity towards insect versus mammalian receptors.19 The simple N-phenyl amide of 5-amino-1,3-dimethyl pyrazole-4-carboxylic acid E has been shown to exhibit antifungal activity.21 The 5-amino-1-pyrAzinyl-3-carboxamido pyrazole derivative F has been recently reported as a potent antibacterial agent with a very broad spectrum (Figure 1).22

Recently, components of the mitotic machinery have been targeted in an attempt to develop novel anticancer agents. These include critical signaling kinases such as the Aurora, polo-like kinases (PLK), and the cyclin-dependent kinases (CDK). Compound G (AZD1152) is the first Aurora B selective inhibitor to enter clinical trials23 (Figure 2). Aurora B facilitates proper bipolar end-on microtubule (MT)-kinetochore attachment,24 participates in spindle assembly checkpoint (SAC) signaling,25 and mediates
chromosome condensation and cohesion. Also, Aurora B relocalizes to the central spindle during late anaphase and to the mid-body during telophase, thereby facilitating cytokinesis. Chemical perturbation of Aurora kinases has proven invaluable in parsing the temporal and spatial functions of each isoform and assessing the therapeutic potential in inhibiting kinase activity in the context of cancer.

On the other hand, thieno[2,3-c]pyrazoles have created great interest in medicinal chemistry due to their broad spectrum of antitumor, antiviral, antimicrobial and anti-inflammatory activities.

In the light of the previous biological importance of pyrazoles and thienopyrazoles, and in continuation of our work for synthesis of new thieno[2,3-c]pyrazoles, we have synthesized a series of novel pyrazolothienotriazines 6-14. Literature survey revealed that the pyrazolothienotriazine system has not been previously synthesized. Therefore, as a result of resistance of some bacterial and fungi strains to the existing antimicrobial therapy, we got interested in the search for syntheses of more effective agents. In addition, non-steroidal anti-inflammatory drugs (NSAIDs), which are widely used for reducing pain and swelling associated with inflammation, represent a research area of continuous development. Hence, the suspected promising biological activities of the pyrazolothienotriazine compounds encouraged us to study the in vitro anti-microbial and in vivo anti-inflammatory activities of some pyrazolothienotriazine heterocycles in comparison with the standard drugs. The obtained results from biological screening demonstrated

Figure 1. Pharmacology active 5-aminopyrazoles.

Figure 2. Anticancer agent AZD 1152.
that most of pyrazolothienotriazine compounds revealed promising antibacterial, antifungal and anti-inflammatory activities, which can be used as potential antibacterial, antifungal and anti-inflammatory drugs.

**Experimental**

All the required chemicals were purchased from Merck, Sigma-Aldrich and Loba chemical companies. The melting points were uncorrected and recorded on a Gallen Kamp electric melting point apparatus. The elemental analyses were carried out at the Micro Analytical Center of Chemistry Department, Assiut University, Egypt. The Fourier transform infrared (FTIR) spectra were recorded using potassium bromide disks on a FT-IR 8201 PC Shimadzu. 

$^1$H and $^{13}$C nuclear magnetic resonance (NMR) spectra were obtained on Varian Mercury VX-300 NMR (300 MHz) and Bruker (400 MHz) spectrometers in CDCl$_3$ and DMSO-d$_6$ using tetramethylsilane (Me$_4$Si) as internal standard and chemical shifts were expressed as ppm. Mass spectra were measured on a Jeol-JMS 600 spectrometer at the Regional Center for Mycology & Biotechnology, Al-Azhar University, Cairo, Egypt. All reactions were monitored by thin layer chromatography (TLC) technique on silica gel coated aluminum sheets (silica gel 60 F$_{254}$, Merck). The chloropyrazolecarbonitrile compound (1) was prepared according to the literature procedure.\textsuperscript{31} Numbering of carbon atoms used in $^{13}$C NMR analyses for compounds 9a, 9c, 13 and 14 is shown in Figure 3.

5-(Cyanomethylthio)-3-methyl-1-phenyl-1$H$-pyrazole-4-carbonitrile (4)

To a stirred suspension of finely powdered sulfur (4.00 g, 0.125 mol) in absolute ethanol (60 mL) in an ice bath, sodium borohydride (4.00 g, 0.105 mol) was added in small portions until all sulfur powder dissolved. Chlorocyanopyrazole 1 (10.00 g, 46 mmol) was added with stirring for additional 1 h. The reaction mixture was heated under reflux at 100 °C for 4 h, followed by cooling. At this stage, the non-isolated sulfanyl sodium salt 3 was formed. After reflux was completed, chloroacetonitrile (3.50 mL, 46 mmol) was added to the reaction mixture and was left overnight with stirring. The solid precipitate formed on cooling was filtered, dried and recrystallized from ethanol as white crystals in 85% (10.00 g) yield; mp 78-80 °C; FTIR (KBr) ν/cm$^{-1}$ 3035 (CH aromatic), 2985-2925 (CH aliphatic), 2275, 2227 (2CN); $^1$H NMR (300 MHz, DMSO-d$_6$) $\delta$ 2.85 (s, 3H, CH$_3$), 3.85 (s, 2H, CH$_2$), 7.60-7.40 (m, 5H, ArH); MS (EI, 70 eV) m/z 254.40 [M]$^+$, 239.24 [M – CH$_3$]$^+$, 177.05 [M – Ph]$^+$; anal. calcd. for C$_{13}$H$_{10}$NO$_4$S (254.32): C, 61.40; H, 3.96; N, 22.03; S, 12.61%. Found: C, 61.23; H, 4.20; N, 21.98; S, 12.75%.

Figure 3. Carbon numbering of compounds 9a, 9c, 13 and 14.
4-Amino-3-methyl-1-phenyl-1H-thieno[2,3-c]pyrazole-5-carbonitrile (5)

Pyrazole 4 (4.00 g, 16 mmol) was heated under reflux at 100 °C in ethanolic sodium ethoxide solution (prepared from 0.50 g of finely divided sodium metal in 20 mL of absolute ethanol) for 10 min. The solid precipitate, which separated out during reflux, was filtered, dried and recrystallized from ethanol:dioxane mixture (2:1) as white crystals in 75% (3.00 g) yield; mp 198-200 °C; FTIR (KBr) ν / cm⁻¹: 3455, 3359, 3229 (NH), 2950, 2890 (CH aliphatic), 2183 (CN); ¹H NMR (300 MHz, DMSO-d₆) δ 2.50 (s, 3H, CH₃), 7.00 (s, 2H, NH₂), 7.30-7.60 (m, 5H, ArH); ¹³C NMR (75 MHz, DMSO-d₆) δ 13 (C7: CH pyrazole), 71.50 (C5), 107.50 (C3a), 116.50 (C9: CN), 122 (C2', C6' aromatic), 126 (C4' aromatic), 127 (C6a), 130.50 (C3', C5' aromatic), 138.50 (C1' aromatic), 144 (C3), 148 (C4); MS (EI, 70 eV) m/z 255.81 [M + 1]; 254.32 [M]; 253.34 [M⁺]; anal. calcd. for C₁₀H₇N₃S (372.45): C, 64.50; H, 3.94; N, 23.45; S, 8.94%. Found: C, 63.63; H, 3.91; N, 23.24; S, 9.04%.

4-Chloro-8-methyl-6-phenyl-6H-pyrazolo[4',3':4,5]thieno[3,2-d][1,2,3]triazine (6)

To a stirred solution of the thienopyrazole 5 (1.30 g, 5 mmol) in a mixture of acetic acid (10 mL) and concentrated HCl 37% (7 mL) at 0-5 °C, sodium nitrite solution (0.40 g in 4 mL H₂O, 10%) was added drop wise within 5 min. After the addition was completed, the reaction mixture was stirred for additional 3 h. The solid product formed was collected, dried and recrystallized from ethanol as orange crystals in 71% (0.78 g) yield; mp 160-162 °C; FTIR (KBr) ν / cm⁻¹: 3030 (CH aromatic), 2922 (CH aliphatic), 1597 (C=N); ¹H NMR (300 MHz, CDCl₃) δ 2.90 (s, 3H, CH₃), 7.40-7.80 (m, 5H, ArH), 8.55 (s, 1H, NH); anal. calcd. for C₁₀H₇ClN₃S (301.75): C, 51.79; H, 2.67; Cl, 11.75; N, 23.21; S, 10.62%. Found: C, 51.78; H, 2.70; Cl, 11.79; N, 23.27; S, 10.52%.

General procedure

To a stirred solution of the chlorotriazine 6 (0.25 g, 0.83 mmol) in absolute ethanol (10 mL), the corresponding primary or secondary amine (2 mmol) and triethyamine (0.1 mL) were added. The reaction mixture was gently refluxed for 2 h. The solid precipitate, which separated out during reflux, was filtered, dried and recrystallized from the proper solvent.

8-Methyl-6-phenyl-4-(p-tolylamino)-6H-pyrazolo[4',3':4,5]thieno[3,2-d][1,2,3]triazine (7b)

Obtained by the reaction with p-toluidine. The solid product formed was recrystallized from ethanol:dioxane mixture (2:1) as greenish white crystals in 78% (0.24 g) yield; mp 128-130 °C; FTIR (KBr) ν / cm⁻¹: 3377 (NH), 3030 (CH aromatic), 2922 (CH aliphatic), 1597 (C=N); ¹H NMR (300 MHz, CDCl₃) δ 2.30 (s, 3H, CH₃ pyrazole), 2.40 (s, 3H, CH₃ p-tolyl), 7.00-7.80 (m, 9H, ArH), 8.80 (s, 1H, NH); anal. calcd. for C₁₀H₁₀N₃S (372.45): C, 64.50; H, 4.33; N, 22.56; S, 8.61%. Found: C, 64.55; H, 4.28; N, 22.60; S, 8.57%.

4-(p-Anisylamino)-8-methyl-6-phenyl-6H-pyrazolo[4',3':4,5]thieno[3,2-d][1,2,3]triazine (7c)

Obtained by the reaction with p-anisidine. The solid product formed was recrystallized from ethanol:dioxane mixture (2:1) as faint brown crystals in 52% (0.17 g) yield; mp 80-82 °C; FTIR (KBr) ν / cm⁻¹: 3400 (NH), 3000 (CH aliphatic), 1595 (C=N), 1244 (C-O); ¹H NMR (300 MHz, CDCl₃) δ 2.70 (s, 3H, CH₃ pyrazole), 3.90 (s, 3H, CH₃ p-anisyl), 7.30-7.80 (m, 9H, ArH), 8.55 (s, 1H, NH); anal. calcd. for C₁₂H₁₀N₂O (388.45): C, 61.84; H, 4.15; N, 21.64; O, 4.12; S, 8.25%. Found: C, 61.94; H, 4.25; N, 21.58; S, 8.18%.

8-Methyl-6-phenyl-4-(piperidin-1-yl)-6H-pyrazolo[4',3':4,5]thieno[3,2-d][1,2,3]triazine (8a)

Obtained by the reaction with piperidine. The solid product formed was collected and recrystallized from ethanol:dioxane mixture (2:1) as faint brown crystals in 71% (0.20 g) yield; mp 100-102 °C; FTIR (KBr) ν / cm⁻¹: 2852, 2932 (CH aliphatic), 3010 (CH aromatic), 1595 (C=N); ¹H NMR (400 MHz, CDCl₃) δ 1.60 (m, 2H, CH₂; C4 piperidinyl), 2.50 (s, 3H, CH₃ pyrazole), 2.78 (m, 4H, 2CH₂; C3, C5 piperidinyl), 3.94 (m, 4H, 2CH₂; C2, C6 piperidinyl), 7.19-7.72 (m, 5H, ArH); anal. calcd. for C₁₀H₁₀N₃S (350.44): C, 61.69; H, 5.18;

N-Carbamimidoyl-4-((8-methyl-6-phenyl-6H-pyrazolo[4',3';4,5]thieno[3,2-d][1,2,3]triazin-4-yl)amino)-p-benzenesulfonamide (9b)

Obtained by the reaction with sulfaguanidine. The solid product formed was recrystallized from ethanol:dioxane mixture (2:1) as faint brown crystals in 57% (0.23 g) yield; mp 144-146 °C; FTIR (KBr) ν / cm⁻¹ 3435, 3400, 3344, 3232 (NH₂, 3NH), 2910 (CH aliphatic), 3050 (CH aromatic), 1597 (C=N), 1442 (SO₂); ¹H NMR (300 MHz, DMSO-d₆) δ 2.50 (s, 3H, CH₃), 5.55 (s, 1H, SO₂NH), 6.50 (s, 2H, NH₂), 7.50-7.70 (m, 9H, ArH), 8.35 (s, 1H, C=NH), 9.10 (s, 1H, NH phenyl); anal. calcd. for C₂₀H₁₉N₅O₂S (479.54): C, 50.09; H, 3.57; N, 26.29; S, 13.37%. Found: C, 50.17; H, 3.68; N, 26.25; S, 13.33%.

8-Methyl-6-phenyl-4-(piperazin-1-yl)-6H-pyrazolo[4',3':4,5]thieno[3,2-d][1,2,3]triazine (8c)

Obtained by the reaction with piperazine. The solid product formed was collected, dried and recrystallized from ethanol:dioxane mixture (2:1) as violet crystals in 52% (0.15 g) yield; mp 122-124 °C; FTIR (KBr) ν / cm⁻¹ 3463, 3400, 3344, 3232 (NH₂, 3NH), 2910 (CH aliphatic), 3050 (CH aromatic), δ 7.30-7.90 (m, 5H, ArH); MS (EI, 70 eV) m/z 352 [M⁺] (42%), 266 [M – morpholine] (16%); anal. calcd. for C₂₀H₁₇N₅OS (352.42): C, 57.94%; H, 4.58%; N, 23.85%; S, 9.20%.

8-Methyl-6-phenyl-6-(morpholin-4-yl)-6H-pyrazolo[4',3':4,5]thieno[3,2-d][1,2,3]triazine (8b)

Obtained by the reaction with morpholine. The solid product formed was recrystallized from ethanol:dioxane mixture (2:1) as violet crystals in 80% (0.20 g) yield; mp 240-242 °C; FTIR (KBr) ν / cm⁻¹ 3435, 3361, 3255 (NH, NH₂ in 80% (0.20 g) yield; mp 122-124 °C; FTIR (KBr) ν / cm⁻¹ 3463, 3400, 3344, 3232 (NH₂, 3NH), 2910 (CH aliphatic), 3050 (CH aromatic), 1597 (C=N), 1442 (SO₂); ¹H NMR (300 MHz, DMSO-d₆) δ 2.50 (s, 3H, CH₃), 5.55 (s, 1H, SO₂NH), 6.50 (s, 2H, NH₂), 7.30-7.60 (m, 9H, ArH), 8.35 (s, 1H, C=NH), 9.10 (s, 1H, NH phenyl); anal. calcd. for C₂₀H₁₉N₅O₂S (479.54): C, 50.09; H, 3.57; N, 26.29; S, 13.37%. Found: C, 50.17; H, 3.68; N, 26.25; S, 13.33%.

N-(4-Hydrazino-8-methyl-6-phenyl-6H-pyrazolo[4',3':4,5]thieno[3,2-d][1,2,3]triazin-4-yl)amino)-N-thiazol-2-yl-p-benzenesulfonamide (9a)

Obtained by the reaction with sulfathiazole. The solid product formed was recrystallized from ethanol:dioxane mixture (2:1) as faint brown crystals in 58% (0.25 g) yield; mp 138-140 °C; FTIR (KBr) ν / cm⁻¹ 3360, 3320 (NH), 3060 (CH aromatic), 2909 (CH aliphatic), 1595 (C=N), 1425 (SO₂); ¹H NMR (300 MHz, DMSO-d₆) δ 2.50 (s, 3H, CH₃), 5.60, 6.70 (2d, J 3.20 Hz, 2H, 2CH thiazolyl), 7.20-7.70 (m, 9H, ArH), 8.80 (s, 1H, NH phenyl), 12.40 (s, 1H, SO₂NH); ¹³C NMR (100 MHz, DMSO-d₆) δ 13 (C9: CH₃ pyrazole), 101.50 (C7a), 104 (C3a), 114 (C17), 117 (C2", C6": benzene sulfonamide), 119 (C2', C6': Ph pyrazole), 123.50 (C4": Ph pyrazole), 126 (C3b), 128 (C8a), 130 (C3', C5": Ph pyrazole), 132 (C4": benzene sulfonamide), 133 (C3", C5": benzene sulfonamide), 136 (C16), 138 (C1": Ph pyrazole), 142 (C1": benzene sulfonamide), 147 (C3), 150 (C7), 166 (C14); anal. calcd. for C₃₀H₂₄N₁₀O₅S₂ (520.60): C, 50.76; H, 3.10; N, 21.52; S, 18.47%. Found: C, 50.67; H, 3.15; N, 21.54; S, 18.40%.

4-Hydrazone-8-methyl-6-phenyl-6H-pyrazolo[4',3':4,5]thieno[3,2-d][1,2,3]triazine (10)

A suspension of the chlorotriazine compound 6 (0.25 g, 0.83 mmol) and hydrazine hydrate (2.00 mL, 0.04 mol) was gently heated in absence of solvent under neat conditions for 5 min, then absolute ethanol (10 mL) was added. The reaction mixture was refluxed for additional 2 h. The solid precipitate formed after cooling was filtered, dried and recrystallized from ethanol as faint brown crystals in 80% (0.20 g) yield; mp 240-242 °C; FTIR (KBr) ν / cm⁻¹ 3463, 3361 and 3255 (NH, NH₂), 2917, 2848 (CH aliphatic), 3030 (CH aromatic), 1596 (C=N); ¹H NMR
(300 MHz, DMSO-d$_6$) $\delta$ 2.60 (s, 3H, CH$_3$), 5.80 (s, 2H, NH$_2$), 7.35-7.80 (m, 5H, ArH), 8.55 (s, 1H, NH); MS (EI, 70 eV) m/z 297 [M]$^+$ (51.89%); anal. calcd. for C$_{14}$H$_{13}$N$_2$S (297.34): C, 52.51; H, 3.73; N, 32.98; S, 10.78%. Found: C, 52.63; H, 3.82; N, 32.84; S, 10.71%.

7-Methyl-9-phenyl-9H-pyrazolo[4',3':4,5]thieno[2,3-e][1,2,4]triazolo[4,3-c][1,2,3]triazine (11)

A mixture of the hydrazino compound 10 (1.00 g, 3.30 mmol) and triethyl orthoformate (3 mL) in the presence of few drops of acetic acid (0.5 mL) were refluxed for 1 h. The solid product that separated out during reflux was filtered, dried and recrystallized from acetic acid as faint brown crystals in 78% (0.80 g) yield; mp > 360 °C; FTIR (KBr) v/cm$^{-1}$: 2924 (CH aliphatic), 3046 (CH aromatic), 1596 (C=N); 1H NMR (300 MHz, DMSO-d$_6$) $\delta$ 2.55 (s, 3H, CH$_3$), 7.40-7.80 (m, 5H, ArH), 8.55 (s, 1H, CH$_3$ pyrazole), 2.55 (s, 3H, CH$_3$ pyrazole), 15 (C15: CH$_3$), 7.35-7.75 (m, 5H, ArH), 8.55 (s, 1H, CH$_3$ pyrazole), 2.75 (s, 3H, CH$_3$ pyrazole), 6.40 (s, 1H, CH$_3$ pyrazole), 7.30-7.70 (m, 5H, ArH); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 10 (C16: CH$_3$ pyrazolyl), 13 (C9: CH$_3$ pyrazole), 15 (C15: CH$_3$ pyrazolyl), 110 (C3a), 114.50 (C13: CH pyrazolyl), 118 (C2', C6': aromatic), 122 (C7a), 124 (C4': aromatic), 126 (C3b), 128.50 (C8a), 132 (C3', C5': aromatic), 137 (C1': aromatic), 142 (C14), 147 (C3), 150 (C12), 163.50 (C7); MS (EI, 70 eV) m/z 361 [M]$^+$ (100%); anal. calcd. for C$_{14}$H$_{13}$N$_2$S (361.43): C, 54.71; H, 2.95; N, 31.90; S, 10.43%. Found: C, 54.67; H, 2.85; N, 31.94; S, 10.54%.

7-Methyl-9-phenyl-2,9-dihydro-3H-pyrazolo[4',3':4,5]thieno[2,3-e][1,2,4]triazolo[4,3-c][1,2,3]triazine (12)

A solution of the hydrazino compound 10 (0.50 g, 1.70 mmol) and carbon disulfide (1 mL) in pyridine (2 mL) was heated on a steam bath for 8 h. The solid precipitate that separated out during reflux was recrystallized from dioxyane as yellow crystals in 74% (0.67 g) yield; mp 210-212 °C; FTIR (KBr) v/cm$^{-1}$: 3400 (NH), 3070 (CH aromatic), 2921, 2850 (CH aliphatic), 3010 (CH aromatic), 1596 (C=N); $^{1}$H NMR (300 MHz, DMSO-d$_6$) $\delta$ 2.50 (s, 3H, CH$_3$), 7.35-7.75 (m, 10H, ArH), 8.30 (s, 1H, CH benzylidene), 9.75 (s, 1H, NH); $^{13}$C NMR (75 MHz, DMSO-d$_6$) $\delta$ 13 (C9: CH$_3$ pyrazolyl), 102 (C7a), 106 (C3a), 110 (C2', C6': aromatic), 115 (C4': Ph pyrazolyl), 119 (C3b), 124 (C8a), 126 (C3', 5': benzylidene), 128 (C2'', C6'': benzylidene), 132 (C3', C5': Ph pyrazolyl), 134 (C4'': benzylidene), 140 (C1'': benzylidene), 142.50 (C1': Ph pyrazolyl), 145 (C12), 147.50 (C3), 152 (C7); anal. calcd. for C$_{15}$H$_{14}$N$_2$S$_2$ (385.45): C, 62.32; H, 3.92; N, 25.44; S, 8.32%. Found: C, 62.37; H, 3.85; N, 25.39; S, 8.39%.

Biological activity tests

In vitro antibacterial assay

All microorganisms used were obtained from the culture collection of Microbiology Department, Faculty of Medicine, Assiut University. Activities of several synthesized compounds against a number of Gram-negative bacterial strains (Haemophilus influenzae, Escherichia coli and Pseudomonas aeruginosa) and a number of Gram-positive bacterial strains (Streptococcus pneumoniae, Bacillus cereus and Bacillus subtilis) were investigated using 5 mL solution of the tested compounds 8a-c in DMSO as a solvent. The synthesized compounds were initially screened by a maximum concentration of 100 µg mL$^{-1}$ in DMSO and a series of antibiotic drugs as references namely: clindamycin, streptomycin, gentamycin, levofloxacin, moxifloxacin and gemifloxacin. The sterile medium (nutrient agar medium, 15 mL) in each Petri dish
was uniformly smeared with cultures of Gram-positive and Gram-negative bacteria. Antibacterial activity of the tested compounds were determined according to the disc diffusion method reported by Kwon-Chung and Bennett using 5 mm diameter filter paper discs loaded with 50 μL of the solution under investigation. The minimum inhibitory concentration (MIC) of each compound was taken as the lowest concentration (mg mL⁻¹) that did not give any visible bacteria growth. The plates were incubated at 37 ± 2 °C for 24 h and the zone of inhibition was determined and listed in Table 1.

In vitro antifungal assay

The fungal strains (Candida albicans, Penicillium sp., Aspergillus fumigatus, Geotrichum candidum, Syncephalastrum racemosum and Trichophyton rubrum) were obtained from some cases of human dermatophytosis (Assiut University Mycological Center, AUMC). The fungal strains were grown in sterilized 9-cm Petri dishes containing Sabouraud dextrose agar (SDA) supplemented with 0.05% of chloramphenicol to suppress bacterial contamination. From these cultures, agar disks (10 mm diameter) containing spores were transferred aseptically to screw-topped vials containing 20 mL sterile distilled water. After shaking, 1 mL samples of the spore suspension were pipetted into sterile Petri dishes, followed by the addition of 15 mL liquefied SDA medium and then left to solidify. The tested compounds 8a-c and the reference compound (ketoconazole) were dissolved in DMSO to give a concentration of 100 μg mL⁻¹. Antifungal activity was determined according to the disc diffusion method reported by Kwon-Chung and Bennett using 5-mm diameter filter paper discs loaded with 50 μL of the solution under investigation (2.0%) and the inoculated plates were incubated at room temperature for 4 days. MIC of each compound was taken as the lowest concentration (mg mL⁻¹) that did not give any visible fungi growth. The zone of inhibition was determined and listed in Table 2.

In vivo anti-inflammatory activity

Anti-inflammatory activity for the newly synthesized compounds 7a-c, 9b and 9c were measured in vivo using carrageenan-induced rat paw edema assay in comparison with indomethacin as a reference drug. The test is

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<td>(4.0)</td>
<td>(5.0)</td>
<td>20 (4.0)</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>16</td>
<td>22</td>
<td>25</td>
<td>moxifloxacin</td>
</tr>
<tr>
<td></td>
<td>(+)</td>
<td>(6.0)</td>
<td>(4.0)</td>
<td>28 (4.0)</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>19</td>
<td>14</td>
<td>19</td>
<td>gemifloxacin</td>
</tr>
<tr>
<td></td>
<td>(+)</td>
<td>(6.0)</td>
<td>(4.0)</td>
<td>25 (4.0)</td>
</tr>
</tbody>
</table>

The amount added of the tested compounds 8a-c and/or the reference antibacterial agent in each pore is 50 μg mL⁻¹.

<table>
<thead>
<tr>
<th>Fungal strain</th>
<th>8a</th>
<th>8b</th>
<th>8c</th>
<th>Ketoconazole</th>
</tr>
</thead>
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<tr>
<td>Candida albicans</td>
<td>14</td>
<td>17</td>
<td>19</td>
<td>21 (4.0)</td>
</tr>
<tr>
<td>Penicillium sp.</td>
<td>13</td>
<td>12</td>
<td>19</td>
<td>18 (4.0)</td>
</tr>
<tr>
<td>Aspergillus fumigatus</td>
<td>13</td>
<td>21</td>
<td>17</td>
<td>22 (4.0)</td>
</tr>
<tr>
<td>Geotrichum candidum</td>
<td>16</td>
<td>15</td>
<td>17</td>
<td>18 (5.0)</td>
</tr>
<tr>
<td>Syncephalastrum racemosum</td>
<td>17</td>
<td>14</td>
<td>14</td>
<td>19 (4.0)</td>
</tr>
<tr>
<td>Trichophyton rubrum</td>
<td>17</td>
<td>14</td>
<td>14</td>
<td>19 (4.0)</td>
</tr>
</tbody>
</table>

The amount added of the tested compounds 8a-c and ketoconazole in each pore is 50 μg mL⁻¹.
based on the pedal inflammation in rat paw induced by sub planter injection of 100 μL of 1% freshly prepared solution of carrageenan in distilled water into the right hind paws of each rat for all the groups; the tested compounds were dissolved in distilled water with sonication. Male adult albino rats (150-200 g) were divided into six groups; each group containing three animals. The thickness of the rat paw edema was measured by a vernier caliper (SMIEC, China). Animals of groups A, B and C, were treated with a single dose of the tested compound, group D was treated with indomethacin drug. Paw thickness were measured just before the carrageenan injection, that is, at “0 hour” and then at 30 min, 1, 2, 3, 4, and 5 h after carrageenan injection. Increasing in paw thickness was measured as a difference in the paw thickness at “0 hour” and at respective hours. The edema was expressed as a mean reduction in paw thickness (mm) after treatment with tested compounds. The percentage of edema inhibition was calculated from the mean effect in the control and treated animals according to the following equation

\[
\text{Edema inhibition (\%)} = \left(1 - \frac{V_t}{V_c}\right) \times 100 \quad (1)
\]

where \(V_t\) is the increase in paw volume of test and \(V_c\) is the increase in paw volume of control group of rats.

Statistical analysis

The results were analyzed by one way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test as a post-test. These analyses were carried out using GraphPad Prism software. The significant differences between groups were accepted at \(p < 0.05^*, 0.01^{**}\) or \(0.001^{***}\), and the data were expressed as a mean ± standard error (SE).

Results and Discussion

In the present work and in continuation of our program for synthesis of novel pyrazolothienotriazine heterocycles that exhibits biological importance, our synthesis is commenced with the preparation of the required substrate starting material 5, which is a useful intermediate for synthesis of fused pyrazolothienotriazines. 4-Amino-3-methyl-1-phenyl-1H-thieno[2,3-c]pyrazole-5-carbonitrile (5) was synthesized by a new method according to literature procedure (Scheme 1). All attempts to displace the chloride ion by the thiol group in the previously prepared 5-chloro-3-methyl-1-phenyl-1H-pyrazole-4-carbonitrile (1) by the reaction with thiourea in ethanol, as with other moieties, to obtain 5-mercapto-3-methyl-1-phenyl-1H-pyrazole-4-carbonitrile (2) failed, giving the chloropyrazole carbonitrile starting material 1. The previous results forced us to search for another method to prepare the target \(o\)-aminothiopyrazole carbonitrile compound 5. The desired results were achieved by the reaction of elemental sulfur with chloropyrazole 1 in the presence of sodium borohydride to give the non-isolated sulfanyl sodium salt 3, which was subjected to react in situ with chloroacetonitrile to afford the pyrazolesulfanyl acetonitrile derivative 4. The latter compound underwent Thorpe-Ziegler cyclization by heating in ethanolic sodium ethoxide solution to afford the amino thienopyrazolecarbonitrile 5.

The chemical structure of compound 5 was elucidated on the basis of its elemental and spectral data. IR spectrum revealed appearance of absorption band at 3455, 3359 and 3229 cm\(^{-1}\) due to NH\(_2\) group. \(^1\)H NMR spectrum showed two singlet signals at \(\delta\) 2.50 and 7.00 ppm, characteristic of CH\(_2\) and NH\(_2\) groups, respectively. \(^13\)C NMR spectrum displayed signals at \(\delta\) 13 and 116.50 ppm attributed to CH\(_{3}\) and CN groups, respectively. Also, mass spectrum displayed a peak at \(m/z\) 254.33 as a molecular ion peak.

Diazotization of the \(o\)-aminothiopyrazole carbonitrile 5 with sodium nitrite solution (10%) in a mixture of acetic acid and concentrated HCl, at room temperature, afforded the newly synthesized chloropyrazolothienotriazine 6. The chemical structure of chlorotriazine 6 was confirmed by IR, \(^1\)H NMR and mass spectra. IR spectrum of compound 6 revealed disappearance of absorption bands characteristic of NH\(_2\) and CN groups and appearance of absorption band at 1593 cm\(^{-1}\) for C=N group. \(^1\)H NMR spectrum of 6 exhibited disappearance of a singlet signal at \(\delta\) 7.00 ppm for NH\(_2\). Also, the mass spectrum of compound 6 displayed a peak at \(m/z\) 301, particular of a molecular ion peak. Furthermore, the chloride ion in compound 6 underwent nucleophilic substitution reactions with various primary and secondary amines upon heating in absence of solvent under neat conditions for a short time, followed by reflux in ethanol to give the \(N\)-substituted aminopyrazolothienotriazine derivatives 7-9 (Scheme 2). Assignment of the chemical structures for the newly synthesized compounds 7-9 were proved from their elemental and spectral analyses. IR spectrum of the phenylamino compound 7a represented absorption band at 3421 cm\(^{-1}\) attributed to NH group. \(^1\)H NMR spectrum showed a singlet signal at \(\delta\) 9.00 ppm for NH group. Also, IR spectrum of piperidinyl compound 8a showed absorption bands at 2852 and 2932 cm\(^{-1}\) for CH aliphatic of piperidine. \(^1\)H NMR spectrum represented multiplet signals at \(\delta\) 1.60-3.94 ppm for five CH\(_2\) groups of piperidine. Moreover, IR spectrum of benzenesulfonamide 9a displayed absorption band at 3330 and 3217 cm\(^{-1}\) for NH and NH\(_2\) groups, and absorption band at 1444 cm\(^{-1}\) for SO\(_2\) group. \(^1\)H NMR spectrum revealed two singlet signals at
Scheme 1. Synthesis of 4-amino-3-methyl-1-phenyl-1H-thieno[2,3-c]pyrazole-5-carbonitrile (5). Reagents and conditions: (i) H₂NCSNH₂/EtOH, reflux 3 h; (ii) S/NaBH₄/EtOH, stirring in an ice bath 1 h; (iii) ClCH₂CN/ EtOH, reflux 2 h, stirring overnight; (iv) EtONa/ EtOH, Δ 10 min.

Scheme 2. Nucleophilic substitution of the chlorotriazine 6 with various primary and secondary amines affording the N-substituted triazine compounds 7-9.
δ 5.90 and 8.70 ppm attributed to NH₂ and NH groups, respectively. ¹³C NMR spectrum of compound 9a revealed signals at δ 114-144 ppm, characteristic of 12 carbon atoms of the two phenyl rings.

Consequently, hydrazinolysis of the chlorotriazine compound 6 with hydrazine hydrate upon heating under neat conditions for a short time, followed by addition of ethanol, furnished the hydrazinopyrazolothienotriazine 10. The latter compound 10 was used as a versatile precursor for synthesis of other heterocyclic rings attached or fused to pyrazolothienotriazine ring system to afford compounds 11-14 (Scheme 3). Thus, the reaction of hydrazino compound 10 with triethylorthoformate, in presence of a catalytic amount of acetic acid, afforded the pyrazolothienotriazolotriazine 11. Also, nucleophilic addition of NH₂ group of hydrazino compound 10 to carbon disulfide, followed by elimination of H₂S, yielded the corresponding triazolothienothione derivative 12. On the other hand, condensation of 10 with acetyl acetone and benzaldehyde gave the corresponding dimethylpyrazolyl 13 and the benzylidenehydrazinotriazine (Schiff’s base) 14, respectively. Formation of compounds 11-14 were assigned by both elemental and spectral analyses. IR spectrum of the triazolothione 12 displayed absorption band at 3400 cm⁻¹ for NH group, while IR spectrum of 13 revealed disappearance of absorption bands of NH and NH₂ groups. ¹H NMR spectrum of 12 showed a singlet signal at δ 9.80 ppm due to NH group.

Biological activities

Antibacterial activity

Antibacterial activity was determined according to the disc diffusion method reported by Kwon-Chung and

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Scheme 3. Synthesis and reactions of the chlorotriazine compound 10 with various reagents forming triazolotriazines 11 and 12, pyrazolyl 13 and benzylidenehydrazinopyrazolothienotriazine 14. Reagents, conditions and yields: (i) NH₂NH₂, fusion 5 min, then reflux in EtOH, 2 h, 80%; (ii) CH(OEt)₃/AcOH, reflux, 1 h, 78%; (iii) CS₂/pyridine, reflux on steam bath, 8 h, 66%; (iv) Ac₂CH/EtOH, reflux, 3 h, 85%; (v) PhCHO/EtOH, piperidine, reflux, 3 h, 74%.
Bennett. The antibacterial screening was measured by the average diameter of the inhibition zones, expressed in mm, and presented in Table 1. It was observed that all the tested compounds 8a-c exhibited a significant antibacterial activity. The piperazinyl compound 8c showed the highest antibacterial activity against all strains of bacteria (Haemophilus influenzae, Escherichia coli, Pseudomonas aeruginosa, Streptococcus pneumoniae, Bacillus cereus and Bacillus subtilis), which its inhibition zones (19-25 mm) were very close to the reference antibiotics zones (20-28 mm). Also, the morpholinyl compound 8b was very effective against Escherichia coli, Pseudomonas aeruginosa, Streptococcus pneumoniae and Bacillus cereus and showed comparable activity with the standard references. At the same time, compound 8b exhibited a moderate activity against Haemophilus influenzae and Bacillus subtilis. The piperidinyl compound 8a revealed a moderate activity against all the tested strains of bacteria as well as the lowest activity among the tested compounds.

Antifungal activity

Antifungal activity was determined according to the disc diffusion method reported by Kwon-Chung and Bennett. The antifungal activity of compounds 8a-c were reported as zones of inhibition and were summarized in Table 2. It revealed that compound 8c exhibited the highest activity against Candida albicans, Penicillium sp., Geotrichum candidum and Trichophyton rubrum, which its inhibition zones (17-22 mm) were very close to ketoconazole ones (18-23 mm). The morpholinyl compound 8b displayed a high antifungal activity against Aspergillus fumigatus as well as a moderate activity against the rest of the fungal strains. Furthermore, compound 8a showed a high activity against Geotrichum candidum and Syncephalastrum racemosum. Also, the piperidinyl compound 8a showed a moderate activity against Candida albicans, Penicillium sp. and Aspergillus fumigatus (Table 2).

Anti-inflammatory activity

The results of anti-inflammatory activity assessment for some of the newly synthesized compounds were summarized in Tables 3 and 4, and they were also presented in Figures 4 and 5. The results were analyzed by one way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test as a post-test. These analyses were carried out using GraphPad Prism software. From the previous results shown in Tables 3 and 4, and Figures 4 and 5, we found that the anti-inflammatory activity of the p-substituted phenylaminotriazines 7b and 7c, and the substituted benzene sulfonamide compounds 9b and 9c, displayed the same effect as indomethacin after 30 min. The p-anisyl amino 7c showed the highest effect of the tested compounds and very close to the effect of indomethacin after 1 h. After 3 h, the p-tolyl amino7b and the p-anisylamino 7c represented a significant anti-inflammatory activity compared to indomethacin. After 4 and 5 h of treatment, the p-tolyl 7b, p-anisyl 7c and the N-carbamimidolyl 9b revealed the highest anti-inflammatory activity and their effects were very close to the effect of indomethacin. Moreover, the phenylamino compound 7a and the thiazolyl p-benzensulfonamide 9c represented low effect on the inflammation on rats during the period of experiment. From the previous results, we can conclude that the p-tolyl and p-anisylamino compounds 7b, 7c and the N-carbamimidolyl 9b were the best anti-inflammatory agents among the tested compounds compared to indomethacin as a reference anti-inflammatory drug.

Conclusions

In the present work, we have provided an easy access for synthesis of novel tricyclic pyrazolothienotriazine 6,
The results of antimicrobial activities assays demonstrated that the tested compounds 8a-c represented significant antibacterial and antifungal activities. On the other hand, the p-tolylamino 7b, p-anisyl amino 7c and 

Table 4. Paw edema inhibition for compounds 7a–c, 9b and 9c

<table>
<thead>
<tr>
<th>Compound</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
<th>240 min</th>
<th>300 min</th>
</tr>
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<tbody>
<tr>
<td>7a</td>
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<td>10.67</td>
<td>24.68</td>
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<tr>
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<td>30.67</td>
<td>45.45</td>
<td>45.45</td>
<td>48.05</td>
</tr>
<tr>
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<td>6.85</td>
<td>20.00</td>
<td>37.33</td>
<td>45.45</td>
<td>45.45</td>
<td>48.05</td>
</tr>
<tr>
<td>9b</td>
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<td>24.00</td>
<td>44.16</td>
<td>45.45</td>
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<tr>
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<td>17.33</td>
<td>38.96</td>
<td>37.66</td>
<td>45.45</td>
</tr>
<tr>
<td>Indomethacin</td>
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<td>20.00</td>
<td>40.00</td>
<td>50.65</td>
<td>51.95</td>
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</tr>
</tbody>
</table>

Figure 4. The relationship between paw edema inhibition with time.

Figure 5. The percentage of edema inhibition with time.

which was used as a versatile precursor for the synthesis of N-alkyl(aryl)amino triazines 7-9 and building new heterocyclic ring systems namely: triazole and pyrazole, attached or fused to the pyrazolothenotriazine moiety.
the N-carbamimidyl 9b showed the highest anti-inflammatory activities compared to indomethacin. From the previous results, we found that most of the examined novel pyrazolothienotriazines exhibited promising antibacterial, antifungal and anti-inflammatory activities, which can be used as potential antibacterial, antifungal and anti-inflammatory drugs.

**Supplementary Information**

Supplementary information (FTIR, 1H NMR, 13C NMR and mass spectral analyses) is available free of charge at http://jbcs.sbq.org.br as PDF file.

**Acknowledgments**

The authors are grateful to Prof Dr Ahmed Abdo Geies, Professor of Organic Chemistry and the President of Assiut University. Also, the authors are grateful to the colleagues in Department of Biology at Faculty of Science, Assiut University, for their kind help in performing the pharmacological screening.

**References**

35. Adeyemi, O. O.; Okpo, S. O.; Ogunti, O. O.; Fitoterapia 2002, 73, 375.
36. GraphPad Prism, version 3.0; GraphPad Software, Inc., San Diego, CA, USA, 1999.