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Synthesis, Antimicrobial Evaluation and Molecular Modeling of Some Novel Phenothiazine Derivatives

Ahmed A. Fadda [a], Ahmed Fekri [a] and Nesma M. Bayoumy [a, b*]

ABSTRACT

Series of novel phenothiazine derivatives with biologically active moieties were synthesized. Treatment of 3-oxo-3-(10H-phenothiazin-10-yl)propanenitrile (**2**) with carbon disulfide gave 3,3-dimercapto-2-(10H-phenothiazine-10-carbonyl)acrylonitrile (**4**) which reacted with different reagents afforded novel fused heterocyclic compounds. The newly synthesized compounds were examined for their antimicrobial activity. Among the tested compounds, compounds **2, 4, 5a,b** and **13** showed the highest antimicrobial activity. Molecular modeling was evaluated and was in agreement with the experimental biological activity results.

Keywords: Phenothiazine; Heterocycles; Haloreagents, Antimicrobial activity, Molecular Modeling.

1. INTRODUCTION

The chemistry involving phenothiazine provides significant related compounds have a variety of biological process. Synthetic phenothiazine and its related compounds are basically effective in the treatment of a number of medical conditions [1, 2]. They possess a wide spectrum of pharmacological/biological activities [3-13] and their several derivatives are in clinical use. Phenothiazine and related compounds have been reported to possess various biological activities such as antimalarial [14], antipsychotropic [15], antimicrobial [16], antitubercular [17, 18], antitumor [19, 20] and analgesic [21]. Phenothiazine itself is found to be a worming agent for livestock. The pesticidal action of phenothiazine results from the fact that they affect the nervous system of insects by inhibiting the breakdown of acetylcholine[2]. The carbonyl and the cyano functions of the cyanoacetamides are readily well positioned for reactions with common reagents to form a variety of heterocyclic derivatives. Cyanoacetamides are highly reactive compounds having an active methylene which can take a part in a variety of condensation and substitution reactions. In the current years, a number of different quantum mechanical theories were ascended, for example the DBG Density Functional Theory (DFT). It was considered an effective computational method for studying organic. The DFT method explained the chemical reactivity and orbitals, energy band gap, electronegativity, dipolemomment. [22]. Therefore, in the present work we aimed to synthesize new phenothiazine derivatives and their evaluation for the anticipated antimicrobial activities.

a. Chemistry Department, Faculty of Science, Mansoura University, Mansoura, Egypt

b. Dental Biomaterials Department, Faculty of Oral and Dental Medicine, Delta University for Science and Technology, Gamasa, Egypt

* Corresponding author. Tel.: 20506432235; fax: 20502246781.

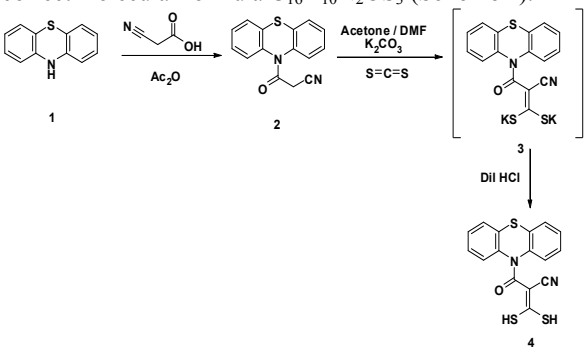
E-mail address: Nesma_Bayoumy@yahoo.com (Nesma M Bayoumy).

2. RESULTS AND DISCUSSION

2.1. CHEMISTRY

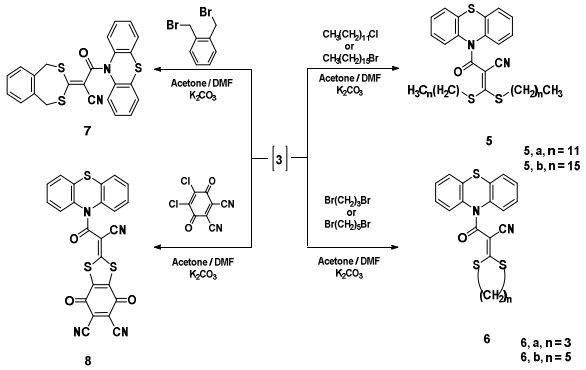
In continuation of our interest, we reported here the behavior of 3-oxo-3-(10H-phenothiazin-10-yl)propane nitrile (**2**) toward some bifunctional reagents as a facile and appropriate route to synthesize some heterocyclic compounds containing phenothiazine moiety.

The cyanoacetamide **2** was synthesized from the reaction of phenothiazine (**1**) with a mixture of cyanoacetic acid and acetic anhydride (**Scheme 1**). Compound **2** was confirmed by both spectral and analytical data. The IR spectrum showed absorption bands at 2259 and 1679 cm^{-1} corresponding to CN and C=O, respectively. The ^{1}H-NMR spectrum showed one singlet signal at δ 3.86 ppm attributable to CH_2 protons of cyanoacetyl group and a multiplet signal at δ 7.46-7.55 ppm due to the aromatic system. Also its mass spectrum showed the molecular ion peak at m/z = 266 $(M)^+$ attributable to its correct molecular formula $C_{15}H_{10}N_2OS$. Reaction of **2** with carbon disulfide afforded the non-isolable intermediate **3** which reacted *in-situ* with dilute hydrochloric acid to afford 3,3-dimercapto-2-(10H-phenothiazine-10-carbonyl)acrylonitrile (**4**). The ^{1}H-NMR spectrum of **4** revealed singlet signal at δ 1.5 ppm attributable to two SH proton. Moreover, the mass spectrum proved the structure through the appearance of the molecular ion peak at m/z = 342 $(M)^+$ which corresponding to the correct molecular formula $C_{16}H_{10}N_2OS_3$ (**Scheme 1**).



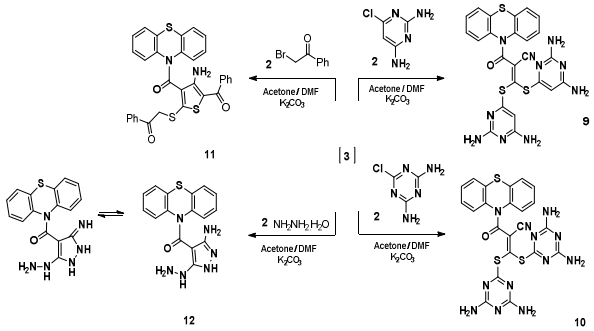
Scheme 1. Reaction of compound **2** with Carbon disulfide.

The intermediate **3** reacted with different halo-alkanes namely, 1-chlorododecane, 1-chlorohexadecane, 1,3-dibromopropane and 1,5-dibromopentane in a stirring mixture of acetone and N,N-dimethylformamide (1:2) containing anhydrous potassium carbonate as a base to afford dithia derivatives **5a,b** and **6a,b**, respectively (**Scheme 2**). Compound **5a** was confirmed by both elemental analysis and spectral data. The ^{1}H-NMR spectrum showed two triplet signals at δ 2.10 and 3.50 ppm corresponding to the two identical CH_3 groups and the two identical S-CH_2 groups, respectively. In addition to, a broad signal at δ 3.30-3.89 ppm due to the two $(CH_2)_{10}$ protons and multiplete signal at δ 7.13-7.43 ppm corresponding to the aromatic system. Moreover, the mass spectrum showed the molecular ion peak at m/z = 679, $(M+1)^+$ and 680 $(M+2)^+$ equivalent to the correct molecular formula $C_{40}H_{58}N_2OS_3$. The ^{1}H-NMR spectrum of **5b** revealed two triplet signals at δ 0.88 and 3.50 ppm corresponding to the two CH_3 and the two SCH_2 groups, respectively, in addition to multiplet signal at δ 1.26-1.42 ppm due to the two $(CH_2)_{14}$ protons and multiplet signal at δ 7.30-7.67 ppm equivalent to the aromatic system. Moreover, the mass spectrum exhibited the molecular ion peak at m/z = 790 $(M)^+$ confirmed the correct molecular formula $C_{48}H_{74}N_2OS_3$. The ^{1}H-NMR spectrum of **6a** displayed triplet signal at δ 2.89 ppm attributable to SCH_2 protons and a broad signal centered at δ 3.50 ppm corresponding to CH_2 protons. The mass spectrum indicated the molecular ion peak at m/z = 382 $(M)^+$ confirmed the molecular formula $C_{19}H_{14}N_2OS_3$ (**Scheme 2**). Compound **6b** was established by both spectral and analytical data. The IR spectrum exhibited absorption frequencies at 2220 and 1698 cm^{-1} corresponding to CN and C=O groups, respectively. The ^{1}H-NMR spectrum showed triplet signal at δ 2.85 ppm corresponding to SCH_2 protons, multiplet at δ 1.32-1.95 ppm corresponding to three CH_2 protons. The mass spectrum showed the molecular ion peak at m/z = 410 $(M)^+$ attributable to the correct molecular formula $C_{21}H_{18}N_2OS_3$. Furthermore, the reaction of intermediate **3** with equimolar amount of dihaloalkanes under the same reaction conditions afforded compounds **7** and **8**. The ^{1}H-NMR spectrum of **7** revealed singlet signal at δ 4.13 ppm corresponding to two SCH_2 protons. Also, the mass spectrum showed the molecular ion peak at m/z= 443 $(M-1)^+$ corresponding to the molecular formula $C_{24}H_{16}N_2OS_3$. Structure **8** revealed absorption frequencies at 2239-2218 and 1720-1698 cm^{-1} attributable to three CN functions and three C=O functions, respectively. Also, the mass spectrum showed the molecular ion peak at m/z = 495 $(M)^+$ corresponding to the molecular formula $C_{24}H_8N_4O_3S_3$ (**Scheme 2**).



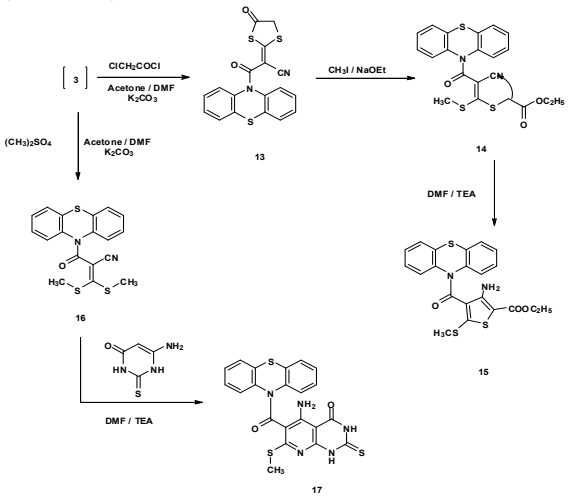
Scheme 2. Reaction of in intermediate **3** with dihalo-reagents.

In addition to that mentioned above, the reaction of intermediate **3** with two moles of 6-chloropyrimidine-2,4-diamine and/or 6-chloro-1,3,5-triazine-2,4-diamine and/or phenacyl bromide in a stirring mixture of acetone and N,N-dimethylformamide (1:2) containing anhydrous potassium carbonate as a base afforded the new phenothiazine derivatives **9-11** (**Scheme 3**). Compounds **9-11** were confirmed on the basis of spectral and analytical data. The IR spectra generally, displayed absorption frequencies at 3398-3210, 2220-2192 and 1644-1721 cm^{-1} corresponding to NH_2, CN and C=O functions, respectively. The ^{1}H-NMR spectrum of compound **9** revealed three singlet signals at δ 4.48, 6.33 and 6.82 ppm corresponding to two C_5-H pyrimidine protons and four NH_2 protons, respectively. Also, the mass spectrum showed the molecular ion peak at m/z = 556 $(M-2)^+$ corresponding to the molecular formula $C_{24}H_{18}N_{10}OS_3$. In additional, the ^{1}H-NMR spectrum of compound **10** revealed two singlet signals at δ 6.94 and 7.12 ppm corresponding to four NH_2 functions. Also, the mass spectrum showed the molecular ion peak at m/z = 560 $(M)^+$ equivalent to the molecular formula $C_{22}H_{16}N_{12}OS_3$. The ^{1}H-NMR spectrum of Compound **11** showed two singlet signals at δ 4.48 and 5.93 ppm consistent to SCH_2 and NH_2 protons, respectively. The mass spectrum revealed the molecular ion peak at m/z = 578 $(M)^+$ due to the molecular formula $C_{32}H_{22}N_2O_3S_3$. On the other hand, the reaction of the intermediate **3** with hydrazine hydrate in in stirring mixture of acetone and N,N-dimethylformamide (1:2) containing potassium carbonate as a base at room temperature, afforded compound **12**. The IR spectrum revealed absorption frequencies at 3410, 3398, 3111, 3096 and 1698 cm^{-1} corresponding to two NH_2, two NH and C=O functions, respectively. Moreover, the ^{1}H-NMR spectrum revealed four singlet signals at δ 5.98, 6.27, 8.46 and 12.33 ppm corresponding to two NH_2 and two NH protons. The mass spectrum displayed the molecular ion peak at m/z = 338 $(M)^+$ corresponding to the molecular formula $C_{16}H_{14}N_6OS$ (**Scheme 3**).



Scheme 3. Reaction of intermediate **3** with haloreagents and hydrazine hydrate.

Furthermore, the reaction of **3** with chloroacetyl chloride in a stirring mixture of acetone and N,N-dimethylformamide (1:2) containing anhydrous potassium carbonate as a base afforded compound **13**. Treatment of compound **13** with methyl iodide in the presence of sodium ethoxide gave the acyclic compound **14** which underwent cyclization afforded compound **15** (**Scheme 4**). Compounds **13-15** were established on the bases of spectral and analytical data. Compound **13** displayed absorption frequencies at 2218, 1710 and 1694 cm^{-1} corresponding to CN and two C=O functions. The ^{1}H-NMR spectrum of **13** revealed singlet signal at δ 3.85 ppm corresponding to CH_2 of the dithiolanone ring. Also, the mass spectrum showed the molecular ion peak at m/z = 381 $(M)^+$ attributable to the correct molecular formula $C_{18}H_{10}N_2O_2S_3$. The ^{1}H-NMR spectrum of compound **14** showed two singlet signals at δ 2.80 and 3.98 ppm attributable to the SCH_3 and SCH_2 protons, triplet signal at δ 1.25 and quartet at δ 4.13 ppm corresponding to the CH_3 and OCH_2 protons, respectively. The mass spectrum showed the molecular ion peak at m/z = 442 $(M)^+$ equivalent to the molecular formula $C_{21}H_{18}N_2O_3S_3$. In addition to, the ^{1}H-NMR spectrum of **15** revealed two singlet signals at δ 2.51 and 5.82 ppm corresponding to the SCH_3 and NH_2 protons. Also, the mass spectrum showed the molecular ion peak at m/z = 442 $(M)^+$ due to the molecular formula $C_{21}H_{18}N_2O_3S_3$ (**Scheme 4**). Also, the intermediate **3** reacted with dimethyl sulfoxide in stirring mixture of acetone and N,N-dimethylformamide (1:2) containing potassium carbonate as a base at room temperature, to afford the S-alkyl derivative **16**. Compound **16** was established by both spectral and analytical data. The ^{1}H-NMR spectrum of compound **16** revealed singlet signal at δ 2.37 ppm corresponding to two SCH_3 protons. The mass spectrum showed the molecular ion peak at m/z = 371 $(M+1)^+$ confirmed the molecular formula $C_{18}H_{14}N_2OS_3$.When compound **16** was heated with 6-aminothiouracil in refluxing DMF afforded the corresponding pyridopyrimidine derivative **17**. Structure **17** was suggested for the reaction product on the basis of elemental analysis and spectral evidence. The IR spectrum revealed absorption bands at 3410, 3120, 3098, 1698 and 1680 cm^{-1} corresponding to NH_2, two NH and two C=O functions. Moreover, the ^{1}H-NMR spectrum revealed four singlet signals at δ 2.54, 6.35, 11,47 and 12.33 ppm corresponding to the SCH_3, NH_2 and two NH protons. In addition to, the mass spectrum showed the molecular ion peak at m/z = 465 $(M)^+$ equivalent to the molecular formula $C_{21}H_{15}N_5O_2S_3$ (**Scheme 4**).



Scheme 4. Reaction of intermediate **3** with chloroacetyl chloride / methyl iodide and dimethyl sulphate/ 6-aminothiouracil.

2.2. Biological Activity

The newly synthesized target compounds were evaluated for their *in vitro* antibacterial activity against *S. aureus* and *B. Subtilis* as example of Gram-positive bacteria and *E. coli* and *P. aeruginosa* as examples of Gram-negative bacteria. They were also evaluated for their *in vitro* antifungal potential against *Candida albicans* and *F. axysporum* fungal strain.

Disk diffusion technique was used for the determination of the antibacterial and antifungal activity. Ampicillin and Colitrimazole were used as reference drugs. The MIC (µg/mL) values were recorded in (**Table 1).** The results depicted in **Table 1** revealed that the most of tested compounds displayed variable inhibitory effects on the growth of the tested Gram-positive and Gram-negative bacterial strains, and also against antifungal strain. Generally most of the tested compounds revealed better activity against the Gram-positive rather than the Gram-negative bacteria.

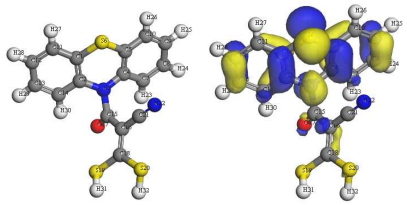
Regarding the structure-activity relationship revealed that compounds **2** and **4** which contain the cyanoacetamide moiety were equipotent to ampicillin in inhibiting the growth of *E. coli* and *P. aeruginosa* (MIC= 125 µg/mL), while their activity were 33% more than of ampicillin against *S. aureus* (MIC= 125 µg/mL). Compound **13** recorded increasing in its activity by 75% more than of ampicillin against *E. coli* (MIC= 31.5 µg/mL) and 50% more than *P. aeruginosa* (MIC = 62.5 µg/mL). Compounds **5a, b** showed increasing in their activity by 66% (MIC= 62.5 µg/mL) and 69%, (MIC= 57.5 µg/mL), respectively more than ampicillin against *S. aureus. While they showed* 50% (MIC = 62.5 µg/mL) more activity than *B.Subtilis*. On the other hand, compound **17** showed very low activity toward most of the tested organisms.

Table 1: Minimal inhibitory concentration (MIC, µg/mL) of some new synthesized compounds.

Compound no.	*MIC in (µg/mL)*					
	Bacteria				*Fungi*	
	Gram- negative bacteria		*Gram- positive bacteria*			
	E. coli	*P. aeruginosa*	*S. aureus*	*B. Subtilis*	*C. albicans*	*F. axysporum*
2	125	125	125	125	250	125
4	125	125	125	125	125	62.5
5a	125	62.5	62.5	62.5	NA	NA
5b	125	62.5	57.5	62.5	NA	NA
6a	NA	NA	125	125	NA	NA
6b	NA	NA	NA	NA	NA	NA
7	125	62.5	125	125	250	125
8	125	125	125	62.5	62.5	31.5
9	125	125	125	125	125	125
10	125	125	187.5	125	125	125
11	125	62.5	125	125	NA	NA
12	250	125	250	125	250	125
13	31.5	62.5	NA	NA	NA	NA
15	125	62.5	187.5	125	3.9	3.2
17	250	250	250	250	250	125
Ampicillin	125	125	187.5	125	NA	NA
Colitrimazole	NA	NA	NA	NA	5.8	4.2

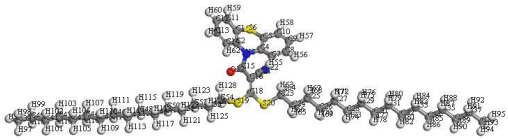
MIC: Minimal inhibitory concentration values with SEM = 0.02.
NAN: o Activity.

Moreover, the experimental results for the newly synthesized compounds were in agreement with the theoretical data. By determining the energy gap (EHOMO - ELUMO) which is an indication for the biological activity. Since molecules with small energy gap were more polarized and reactive than hard one because they easily offer electrons to an acceptor. Also, the low values of energy gap may be due to the groups entering into conjugation in those soft molecules [23-25]. Therefore, the energy gap for the three highest antimicrobial activity compounds (**4, 5b** and **13**; **Table 1**) were determined (**Table 2**). The energy gap of compounds **5b** and **13** (**Figures 2** and **3**, respectively) were smaller than that of compound **4** (**Figure 1**) indicating the ease of charge transfer in compounds **5b** and **13** which enhanced the biological activity of them than compound **4** and are in agreement with the experimental data in **Table 1**. **Table 2** showed the HOMO-LUMO Energy gap, dipole moment and binding energy of compounds **5b** and **13** computed using DFT utilizing BLYP energy functional and the basis set DNP. Compound **13** has the most potent activity against *E. coli* and *P. aeruginosa* as gram-negative bacteria. This could be related the lowest value of dipole moment for compound **13** than compounds **4** and **5b**. The inverse correlation between the dipole moment and the activity of the compound **13** could be clarified in the concept that decreasing the dipole moment will decrease the polarity and increase the lipophilic nature of the compound which favoured its permeation more efficiently through the lipid layer of the microorganism [26]. While, the negative results can be attributed either to the inability of these compounds to diffuse through the cell wall of the bacterium and hence unable to interfere with its biological activity or they can diffuse and inactivated by unknown cellular mechanism i.e. bacterial enzymes [27]. Also, the isolated compound **13** showed large values of binding energy than the compound **4** and **5b** that improved the stability of this compound [28]

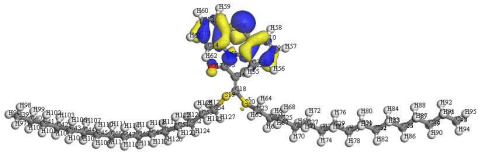


(a) **(b)**

Figure 1: (**a**) molecular geometry of compound **4** and (**b**) calculated HOMO superimposed on the isosurface of the molecular geometry of compound **4**.

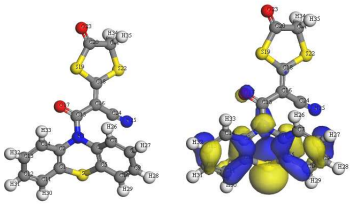


(a)



(**b**)

Figure 2: (**a**) molecular geometry of conformer **5b** and (**b**) calculated HOMO superimposed on the isosurface of the molecular geometry of conformer **5b**.



(a) **(b)**

Figure 3: (**a**) molecular geometry of conformer **13** and (**b**) calculated HOMO superimposed on the isosurface of the molecular geometry of conformer **13**.

Table 2: Calculated total energy and binding energy (BLYP/DNP) for compounds (**4**, **5b** and **13** using DFT.

Compound	HOMO-LUMO Energy gap (eV)	Dipole moment Debye	Binding energy, Hartree
4	-1.877	2.1151	-6.3781553
5b	-2.167	5.6224	-6.7501331
13	-2.488	1.9024	-20.8448839

Regarding the activity of the compounds, against antifungal strains, the results revealed that compound **15** was 32.7 % (MIC= 3.9 µg/mL) and 24% (MIC= 3.2 µg/mL) higher than colitrimazole in inhibiting the growth of *C. albicans* and *F. axysporum*, respectively. Compounds **2, 4, 7, 8, 9, 10, 12** and **17** exhibited moderate growth inhibitory activity against *C. albicans* and *F. axysporum* as revealed from their MIC values (MIC= 31.5 - 250 µg/mL).

The tested compounds were more active against Gram-positive than Gram-negative bacteria, it may be concluded that the antimicrobial activity of the compounds is related to cell wall structure of the bacteria. It is possible because the cell wall is essential to survival of bacteria and some antibiotics are able to kill bacteria by inhibiting a step in the synthesis of peptidoglycan. Gram-positive bacteria possess a thick cell wall containing many layers of consisting of a few layers of peptidoglycan surrounded by a second lipid membrane containing peptidoglycan and teichoic acids, but in contrast, Gram-negative bacteria have a relatively thin cell wall lipopolysaccharides and lipoproteins. These differences in cell wall structure can produce differences in antibacterial susceptibility and some antibiotics can kill only Gram-positive bacteria and are inactive against Gram-negative pathogens [29].

In conclusion, the present study describes the synthesis and investigates the antimicrobial activities of some new functionalized phenothiazine derivatives. Also, the HOMO, LUMO, dipole moment and charges on the atoms were assisted to confirm the geometry of the isolated compounds. Moreover, the compounds were screened for antimicrobial activity. Regarding the structure activity relainship compounds **5a, b** exhibited the highest biological activity toward gram positive bacteria, while compound **13** was toward gram negative bacteria. On the other hand, compound **15** showed the highest activity against pathogenic fungi by *C. albicans* and *F. axysporum* using MICs method.

2.3. Molecular modelling

All molecular geometries were fully optimized using DMOL3 program [30] implemented in Materials Studio 7.0 (Accelerys,CA) [31]. DMOL3, BLYP energy functional with the basis set DNP were performed. DFT semi-core pseudopods calculations (dspp) were performed with the double numerical basis sets plus polarization functional (DNP). This basis set was comparable in quality to Gaussian 6-31G sets [32]. Delley et al. showed that the DNP basis sets were more accurate than Gaussian basis sets of the same size [33]. The RPBE functional [34] was so far the best exchange–correlation functional [35, 36], based on the generalized gradient approximation (GGA), was employed to take account of the exchange and correlation effects of electrons.

3. Experimental

3.1. Instruments

All melting points are recorded on Gallenkamp electric melting point apparatus and are uncorrected. The IR spectra ν/cm-1 (KBr) were recorded on Perkin Elmer Infrared Spectrophotometer Model 157, Grating. The ^{1}H-NMR spectra were run on Varian Spectrophotometer at 300 and 75 MHz, respectively, using tetramethylsilane (TMS) as an internal reference and DMSO-d6 as solvent. The mass spectra (EI) were recorded on 70 eV with Kratos MS equipment and/or a Varian MAT 311 A Spectrometer. Elemental analyses (C, H and N) were carried out at the micro analytical center of Cairo University, Giza, Egypt, the results were found to in good agreement (±0.3%) with the calculated values.

3.1.1. Synthesis of 3-oxo-3-(10H-phenothiazin-10-yl)propanenitrile (2)

Phenothiazine (**1**) (10 mmol) was fused with equimolar amount of cyanoacetic acid (10 mmol) in acetic anhydride at 80-85^{0}C in an boiling water-bath for 40 min. leave the reaction mixture for 1hr then filter off. The solid product was washed with ethanol (20 mL) to produce 2 as white crystals. Yield: 90 % M.p. : 215-217 ^{0}C. IR (KBr): ν/cm^{-1}=2259 (C=N), 1679 (C=O); ^{1}H-NMR (DMSO-d6) δ (ppm) =3.86 (s, 2H, CH_2), 7.49-7.55 (m, 8H, Ar-H); MS (EI, 70 eV): m/z (%) = 266 (M^+; 100), 198 (98), 167 (78), 96(88); Chemical Formula: $C_{15}H_{10}N_2OS$: (266.05):; Calcd.: C, 67.65; H, 3.78; N, 10.52% Found: C, 67.82; H, 3.54; N, 10.91%.

3.1.2. Synthesis of 3,3-dimercapto-2-(10H-phenothiazine-10-carbonyl)acrylonitrile (4)

To a stirred suspension of potassium carbonate in a mixture of acetone and DMF (2:1), N-cyanoacetyl phenothiazine (**2**) (10 mmol) was added. To the resulting solution the carbon disulfide (10 mmol) was added and the reaction mixture stirred for 24 h at room temperature to give nonisolable intermediate **3**. The reaction mixture was poured onto crushed ice and neutralized by dilute HCl. The obtained solid product was collected by filteration, washed, dried and crystallized from ethanol to give compound **4**.

White crystals; yield 82% m.p. 218^{0}C; IR (KBr): ν/cm^{-1} = 2218 (CN), 1692 (C=O), 1310 (C=S); ^{1}H-NMR (DMSO-d6) δ (ppm) =1.5 (s, 1H, SH), 7.40-7.70 (m, 8H, Ar-H); MS (EI, 70 eV): m/z (%) = 342 (M^+; 65), 241 (34), 187 (45), 172 (26), 93(100); Chemical Formula: $C_{16}H_{10}N_2OS_3$: (342.00); Calcd.: C, 56.12; H, 2.94; N, 8.18%; Found: C, 56.17; H, 2.89; N, 8.24%.

General procedure for the synthesis of compounds 5a, b

To a stirred mixture of **3** (10 mmol), 1-chlorododecane and / or 1-chlorohexadecane (10 mmol) was added to the mixture and stirred for several 4 h. The reaction mixture was poured onto crushed ice and neutralized by dil HCl. The obtained solid product was collected by filteration, washed, dried and crystallized from ethanol to give compounds **5a,b**.

3.1.3. Synthesis of 3,3-bis(dodecylthio)-2-(10H-phenothiazine-10-carbonyl)acrylonitrile (5a)

paj crystals; yield 76% m.p. 227^{0}C; IR (KBr): ν/cm^{-1}= 2216 (CN), 1720 (C=O), 1317 (C=S); ^{1}H-NMR (DMSO-d6) δ (ppm) =2.10 (t, 6H, 2CH_3), 3.49 (t, 4H, 2SCH_2), 3.50 (br., 40H, 2$(CH_2)_{10}$), 7.13-7.43 (m, 8H, Ar-H); MS (EI, 70 eV): m/z (%) = 680 (M^++2) (18), 391 (34), 327 (21), 276 (47), 77 (100); Chemical Formula: $C_{40}H_{58}N_2OS_3$: (678.37): Calcd.: C, 70.75; H, 8.61; N, 4.13%; Found: C, 70.82; H, 8.58; N, 4.19%.

3.1.4. Synthesis of 3,3-bis(hexadecylthio)-2-(10H-phenothiazine-10-carbonyl)acrylonitrile (5b)

White crystals; yield 72% m.p. 218^{0}C; IR (KBr): ν/cm^{-1}= 2220 (CN), 1680 (C=O), 1310 (C=S).; ^{1}H-NMR (DMSO-d6) δ (ppm) = 0.88 (t, 6H, 2CH_3), 1.26-1.42 (m, 56 H, 2$(CH_2)_{14}$), 3.50 (t, 4H, 2SCH_2), 7.30-7.67 (m, 8H, Ar-H); MS (EI, 70 eV): m/z (%) = 790 (M^+) (65), 636 (22), 520 (28), 401 (18), 327 (48), 276 (34), 180 (17), 77 (100); Chemical Formula: $C_{48}H_{74}N_2OS_3$: (790.50): Calcd.: C, 72.86; H, 9.43; N, 3.54%; Found: C, 72.92; H, 9.39; N, 3.58%.

General procedure for the synthesis of compounds 6a, b

To a stirred mixture of **3** (10 mmol), 1,3-dibromopropane and / or 1,5-dibromopentane (10 mmol) was added to the mixture and stirred for several 4 h. The reaction mixture was poured onto crushed ice and neutralized by dil HCl. The obtained solid product was collected by filteration, washed, dried and crystallized from ethanol to give compounds **6a,b**.

3.1.5. Synthesis of 2-(1,3-dithian-2-ylidene)-3-oxo-3-(10H-phenothiazin-10-yl)propane nitrile (6a)

Orange crystals; yield 68% m.p. 289^{0}C; IR (KBr): ν/cm^{-1}= 2221 (CN), 1712 (C=O), 1314 (C=S); ^{1}H-NMR (DMSO-d6) δ (ppm) = 2.89 (t, 4H, 2SCH_2), 3.50 (br., 2H, CH_2), 6.74-7.67 (m, 8H, Ar-H); MS (EI, 70 eV): m/z (%) = 382 (M^+) (18), 319 (100), 289 (34), 160 (28), 121 (18), 77 (34); Chemical Formula: $C_{19}H_{14}N_2OS_3$: (382.03): Calcd.: C, 59.66; H, 3.69; N, 7.32%; Found: C, 59.72; H, 3.72; N, 7.38%.

3.1.6. Synthesis of 2-(1,3-dithiocan-2-ylidene)-3-oxo-3-(10H-phenothiazin-10-yl)propanenitrile (6b).

Yellow crystals; yield 62% m.p. 272^{0}C; IR (KBr): ν/cm^{-1}= 2220 (CN), 1698 (C=O), 1321 (C=S); ^{1}H-NMR (DMSO-d6) δ (ppm) = 1.32-1.95 (m, 6H, 3CH_2), 7.32-7.72 (m, 8H, Ar-H); MS (EI, 70 eV): m/z (%) = 410 (M^+) (21), 346 (27), 279 (42), 175 (58), 77 (100), 65 (78); Chemical Formula: $C_{21}H_{18}N_2OS_3$: (410.06): Calcd.: C, 61.43; H, 4.42; N, 6.82%; Found: C, 61.46; H, 4.38; N, 6.86%.

General procedure for the synthesis of compounds 7 and 8.

To a stirred mixture of **3** (10 mmol), 1,2-bis(bromomethyl)benzene (10 mmol) and / or 4,5-dichloro-3,6-dioxocyclohexa-1,4-diene-1,2-dicarbonitrile (10 mmol) was added to the mixture and stirred for several 4 h. The reaction mixture was poured onto crushed ice and neutralized by dil HCl. The obtained solid product was collected by filteration, washed, dried and crystallized from ethanol to give compounds **7** and **8**, respectively.

3.1.7. Synthesis of 2-(1,5-dihydrobenzo[e][1,3] dithiepin-3-ylidene)-3-oxo-3-(10H-phenothia zin-10-yl)prop anenitrile (7).

Brown crystals; yield 68% m.p. 345^{0}C; IR (KBr): ν/cm^{-1}= 2222 (CN), 1698 (C=O); ^{1}H-NMR (DMSO-d6) δ (ppm) = 4.13 (s, 4H, 2SCH_2), 7.02-7.78 (m, 12H, Ar-H); MS (EI, 70 eV): m/z (%) = 443 (M^+-1) (18), 304 (58), 246 (60), 155 (42), 123 (100); Chemical Formula: $C_{24}H_{16}N_2OS_3$: (444.04); Calcd.: C, 64.84; H, 3.63; N, 6.30%; Found: C, 64.92; H, 3.68; N, 6.26%.

3.1.8. Synthesis of 2-(1-cyano-2-oxo-2-(10H-phenothiazin-10-yl)ethylidene)-4,7-dioxo-4,7-dihydro benzo[d][1,3]dithiole-5,6-dicarbo nitrile (8)

Paj crystals; yield 72% m.p. 295^{0}C**;** IR (KBr): ν/cm^{-1}= 2239, 2220, 2218 (3CN), 1723, 1720, 1698 (3C=O); ^{1}H-NMR (DMSO-d_6) δ (ppm) = 6.89-7.02 (m, 8H, Ar-H); MS (EI, 70 eV): *m/z* (%) = 495 (M^+) (48), 454 (64), 380 (72), 278 (68), 224 (60), 171 (100), 132 (74); Chemical Formula: $C_{24}H_8N_4O_3S_3$: (495.98)**;** Calcd.: C, 58.06; H, 1.62; N, 11.28%.**;** Found: C, 57.96; H, 1.89; N, 11.32%.

General procedure for the synthesis of compounds 9, 10, 12 and 16.

To a stirred mixture of **3** (10 mmol), 6-chloropyrimidine-2,4-diamine (20 mmol) and / or 6-chloro-1,3,5-triazine-2,4-diamine (20 mmol) and / or hydrazine hydrate (20 mmol) and / or dimethyl sulfoxide (20 mmol) was added to the mixture and stirred for several 4 h. The reaction mixture was poured onto crushed ice and neutralized by dil HCl. The obtained solid product was collected by filteration, washed, dried and crystallized from ethanol to give compounds **9, 10, 12** and **16**, respectively.

3.1.9. Synthesis of 3,3-bis((2,6-diaminopyrimidin-4-yl)thio)-2-(10H-phenothiazine-10-carbonyl)acrylo nitrile (9)

Beige crystals; yield 60% m.p. 289^{0}C; IR (KBr): ν/cm^{-1}= 3397, 3315, 3309, 3210 (4NH_2), 2192 (CN), 1644 (C=O).; ^{1}H-NMR (DMSO-d6) δ (ppm) = 4.48 (s, 2H, C_5-H pyrimidine), 6.33 (s, 4H, 2NH_2), 6.82 (s, 4H, 2NH_2), 7.70-7.78 (m, 8H, Ar-H); MS (EI, 70 eV): m/z (%) = 556 (M^+-2) (52), 473 (18), 304 (59), 246 (78), 155 (48), 123 (100), 91 (78); Chemical Formula: $C_{24}H_{18}N_{10}OS_3$: (558.08): Calcd.: C, 51.60; H, 3.25; N, 25.07%; Found: C, 51.59; H, 3.22; N, 24.97%.

3.1.10. Synthesis of 3,3-bis((4,6-diamino-1,3,5-triazin-2-yl)thio)-2-(10H-phenothiazine-10-carbonyl)acrylo nitrile (10).

White crystals; yield 58% m.p. 335^{0}C; IR (KBr): ν/cm^{-1}= 3421, 3402, 3398, 3318 (4NH_2), 2198 (CN), 1689 (C=O); ^{1}H-NMR (DMSO-d6) δ (ppm) = 6.94 (s, 4H, 2NH_2), 7.12 (s, 4H, 2NH_2), 7.30-7.69 (m, 8H, Ar-H); MS (EI, 70 eV): m/z (%) = 560 (M^+) (38), 453 (28), 332 (29), 266 (36), 198 (100), 156 (32), 80 (67); Chemical Formula: $C_{22}H_{16}N_{12}OS_3$: (560.07): Calcd.: C, 47.13; H, 2.88; N, 29.98%; Found: C, 47.29; H, 2.94; N, 30.02%.

3.1.11. Synthesis of (5-hydrazinyl-3-imino-2,3-dihydro-1H-pyrazol-4-yl)(10H-phenothiazin-10-yl)methan one (12)

Beige crystals; yield 63% m.p. 316^{0}C; IR (KBr): ν/cm^{-1} = 3410, 3398 (2 NH_2), 3096 (NH), 1698 (C=O). ^{1}H-NMR (DMSO-d6) δ (ppm) = 5.98 (s, 2H, NH_2), 6.27 (s, 2H, NH_2), 7.34-7.75 (m, 8H, Ar-H), 8.46 (s, 1H, NH), 12.33 (s, 1H, NH); MS (EI, 70 eV): m/z (%) = 338 (M^+) (72), 289 (87), 250 (100), 182 (62), 75 (78); Chemical Formula: $C_{16}H_{14}N_6OS$: (338.03): Calcd.: C, 58.35; H, 3.81; N, 7.56%; Found: C, 58.42; H, 3.79; N, 7.58%.

3.1.12. Synthesis of 3,3-bis(methylthio)-2-(10H-pheno thiazine-10-carbonyl) acrylonitrile (16)

Red crystals; yield 63% m.p. 316^{0}C; IR (KBr): ν/cm^{-1}= 2228 (CN), 1680 (C=O); ^{1}H-NMR (DMSO-d6) δ (ppm) = 2.37 (s, 6H, 2SCH_3), 7.34-7.75 (m, 8H, Ar-H); MS (EI, 70 eV): m/z (%) = 371 (M^+) (12), 346 (58), 318 (49), 262 (12), 174 (69), 69 (100); Chemical Formula: $C_{18}H_{14}N_2OS_3$: (370.03): Calcd.: C, 58.35; H, 3.81; N, 7.56%.; Found: C, 58.42; H, 3.79; N, 7.58%.

3.1.13. Synthesis of 2-(3-amino-5-((2-oxo-2-phenyl ethyl)thio)-4-(10H-phenothiazine-10-carbonyl) thiophen-2-yl)-1-phenylethan-1-one (11)

To a stirred mixture of **3** (10 mmol), phenacyl bromide (20 mmol) was added to the mixture and stirred for several 4 h. The reaction mixture was refluxed in DMF which contain a catalytic amount of TEA for 6 h. The reaction mixture allowed to cool to room temperature then poured onto cold-ice water and neutralized with conc. HCl. The solid obtained was filtered off and recrystallized from ethanol yielded compounds **11**.

Brown crystals; yield 68% m.p. 345^{0}C; IR (KBr): ν/cm^{-1}= 3398 (NH_2), 1721, 1712, 1689 (3C=O); ^{1}H-NMR (DMSO-d6) δ (ppm) = 4.48(s, 2H, SCH_2), 5.93 (s, 2H, NH_2), 7.41-7.75 (m, 18H, Ar-H); MS (EI, 70 eV): m/z (%) = 578 (M^+) (24), 169 (37), 135 (23), 112 (48), 92 (100); Chemical Formula: $C_{32}H_{22}N_2O_3S_3$: (578.08); Calcd.: C, 66.41; H, 3.83; N, 4.84%; Found: C, 66.46; H, 3.79; N, 4.88%.

3.1.14. Synthesis of 3-oxo-2-(4-oxo-1,3-dithiolan-2-ylidene)-3-(10H-phenothiazin-10-yl)propanenitrile (13)

To a stirred mixture of **3** (10 mmol), phenacyl bromide (20 mmol) was added to the mixture and stirred for several 4 h. The reaction mixture was poured onto crushed ice and neutralized by dil HCl. The obtained solid product was collected by filteration, washed, dried and crystallized from ethanol to give compound **13**.

Orange crystals; yield 70% m.p. 226^{0}C; IR (KBr): ν/cm^{-1}= 2218 (CN), 1710 (C=O); ^{1}H-NMR (DMSO-d6) δ (ppm) = 3.85 (s, 2H, CH_2), 7.49-7.67 (m, 18H, Ar-H); MS (EI, 70 eV): m/z (%) = 318 (M^+) (18), 281 (58), 217 (18), 179 (28), 154 (78), 70 (100); Chemical Formula: $C_{18}H_{10}N_2O_2S_3$: (381.99): Calcd.: C, 56.53; H, 2.64; N, 7.32%; Found: C, 56.62; H, 2.81; N, 7.28%.

3.1.15. Synthesis of ethyl-2-((2-cyano-1-(methylthio)-3-oxo-3-(10H-phenothiazin-10-yl)prop-1-en-1-yl) thio)acetate (14)

To solution of sodium ethoxide (prepared from 10 mmol sodium metal in 10 ml ethanol), methyl iodide (10 mmol) was added to compound **13** (10 mmol). The reaction mixture was poured onto crushed ice and neutralized by dilute HCl. The obtained solid product was collected by filteration, washed, dried and crystallized from ethanol to give compound **14**.

Orange crystals; yield 62% m.p. 265^{0}C; IR (KBr): ν/cm^{-1} = 2220 (CN), 1710, 1698 (2C=O); ^{1}H-NMR (DMSO-d6) δ (ppm) = 1.25 (t, 3H, CH_3), 2.80 (s, 3H, SCH_3), 3.98 (s, 2H, SCH_2), 4.13 (q, 2H, OCH_2), 7.32-7.81 (m, 8H, Ar-H); MS (EI, 70 eV): m/z (%) = 442 (M^+) (18), 364 (37), 286 (18), 213 (29), 154 (78), 77 (100); Chemical Formula: $C_{21}H_{18}N_2O_3S_3$: (442.05): Calcd.: C, 56.99; H, 4.10; N, 6.33%; Found: C, 57.01; H, 4.14; N, 6.28%.

3.1.16. Synthesis of ethyl 3-amino-5-(methylthio)-4-(10H-phenothiazine-10-carbonyl)thiophene-2-carboxylate (15)

Compound **14** (10 mmol) was refluxed in DMF which contain a catalytic amount of TEA for 6 h. The reaction mixture allowed to cool to room temperature then poured onto cold-ice water and neutralized with conc. HCl. The solid obtained was filtered off and recrystallized from ethanol yielded compounds **15**.

Brown crystals; yield 60% m.p. 290^{0}C; IR (KBr): ν/cm^{-1} = 3401 (NH_2), 1720, 1698 (2C=O); ^{1}H-NMR (DMSO-d6) δ (ppm) = 1.34 (t, 3H, CH_3), 2.51 (s, 3H, SCH_3), 4.40 (q, 2H, OCH_2), 5.82 (s, 2H, NH_2), 7.30-7.67 (m, 8H, Ar-H); MS (EI, 70 eV): m/z (%) = 442 (M^+) (21), 364 (37), 316 (18), 288 (48), 211 (59), 169 (76), 92 (100); Chemical Formula: $C_{21}H_{18}N_2O_3S_3$: (442.05): Calcd.: C, 56.99; H, 4.10; N, 6.33%; Found: C, 57.01; H, 4.14; N, 6.28%.

3.1.17. Synthesis of 5-amino-7-(methylthio)-6-(10H-phenothiazine-10-carbonyl)-2-thioxo-2,3-dihydro pyrido[2,3-d]pyrimidin-4(1H)-one (17)

A mixture of compound **16** (10 mmol) and 6-aminothiouracil (10 mmol) were refluxed in DMF which contain a catalytic amount of TEA for 6 h. The reaction mixture allowed to cool to room temperature then poured onto cold-ice water and neutralized with conc. HCl. The solid obtained was filtered off and recrystallized from ethanol yielded compounds **17**.

Brown crystals; yield 78% m.p. over 350^{0}C; IR (KBr): ν/cm^{-1}= 3410 (NH_2), 3120, 3098 (2NH), 1698, 1680 (2C=O); ^{1}H-NMR (DMSO-d6) δ (ppm) = 2.54 (s, 3H, SCH_3), 6.35 (s, 2H, NH_2), 7.29-7.68 (m, 8H, Ar-H), 11.47 (s, 1H, NH), 12.33 (s, 1H, NH); MS (EI, 70 eV): m/z (%) = 465 (M^+) (48), 262 (12), 217 (18), 174 (58),

108 (39), 69 (100); Chemical Formula: $C_{21}H_{15}N_5O_2S_3$: (465.04); Calcd.: C, 54.18; H, 3.25; N, 15.04%; Found: C, 54.21; H, 3.29; N, 14.98%.

3.2. Biological Activity

Antibacterial assay

Antibacterial studies of the newly synthesized compounds were carried out against the representative panel of Gram-positive *S. aureus* and *B. Subtilis* and Gram-negative *E. coli* and *P. aeruginosa*. The activity of compounds was determined as per National Committee for Clinical Laboratory Standards (NCCLS) protocol using Müller-Hinton Broth. Primary screening was done first for antibacterial activity in six sets against *E. coli*, *S. aureus* at different concentrations of 1000, 500, 250 µg/mL. The compounds found to be active in primary screening were similarly diluted to obtain 200, 125, 100, 62.5, 50, 25, 12.5 µg/mL concentrations of secondary screening to test in a second set of dilution against all microorganisms. Inoculum size for test strain was adjusted to 106 CFU/mL (Colony Forming Unit per milliliter) by comparing the turbidity (turbidimetric method). Müller-Hinton Broth was used as nutrient medium to grow and dilute the compound suspension for test organisms. 2% DMSO was used as a diluent/vehicle to obtain the desired concentration of synthesized compounds and standard drugs to test upon standard microbial strains. Synthesized compounds were diluted to 1000 µg/mL concentration as stock solution. The control tube containing no antibiotic was immediately sub cultured [before inoculation] by spreading a lapful evenly over quarter of plate of medium suitable for the growth of test organisms. The culture tubes were then incubated for 24 h at 36 °C and the growth was monitored visually and spectrophotometrically. 10 µg/mL suspensions were further inoculated on an appropriate media and growth was noted after 24 h and 48 h. The lowest concentration (highest dilution) required to arrest the growth of bacteria was regarded as minimal inhibitory concentration (MIC) i.e the amount of growth from the control tube before incubation (which represents the original inoculum) was compared. Solvent had no influence on strain growth. The result of this was greatly affected by the size of inoculum. The test mixture should contain 106 CFU/mL organisms. DMSO and sterilized distilled water were used as negative control while Ampicillin antibiotic (I U strength) was used as positive control. Standard drug used in the present study was "ampicillin" for evaluating antibacterial activity.

Antifungal assay

The newly prepared compounds were screened for their antifungal activity as primary screening in six sets against *Candida albicans* and *F. axysporum* at various concentration of 1000, 500, 250 µg/mL. The primary active compounds were similarly diluted to obtain 200, 125, 100, 62.5, 50, 25, 12.5 µg/mL concentrations of secondary screening to test in a second set of dilution against fungi. The fungal activity of each compound was compared with Colitrimazole as a standard drug, which showed (MIC= 5.8 µg/mL) against C. albicans. For fungal growth, in the present protocol, we have used Sabourauds dextrose broth at 28 °C in aerobic condition for 48 h. DMSO and sterilized distilled water were used as negative control while Colitrimazole (I U strength) was used as positive control.

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