Regular Article

Uses of 1-(3-Cyano-4,5,6,7-tetrahydrobenzo[*b*]-thiophen-2-yl)-3-dodecanoylthiourea as a Building Block in the Synthesis of Fused Pyrimidine and Thiazine Systems

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The reaction of lauroyl isothiocyanate and 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carbonitrile was used to synthesize the title compound 2. Compound 2 could serve as the main building block in the synthesis of many target heterocyclic systems. Various fused pyrimidines were synthesized in the reactions of compound 2 with sodium ethoxide, hydrazine hydrate, phenyl hydrazine, ethyl carbazate, thiourea, and/or 2-aminothiophenol. The structures of the synthesized compounds were confirmed by microanalytical and spectral data.

Key words lauroyl isothiocyanate; pyrimidine derivative; fused system; 2-amino-4,5,6,7-tetrahydrobenzo[*b*]-thiophene-3-carbonitrile

Pyrimidine and fused heterocyclic pyrimidine derivatives have received significant attention over the past few years owing to their therapeutic and pharmacological properties.[1–6] In general, heterocycles encompassing a pyrimidine moiety have found applications in a wide spectrum of biological area.[7–9] Such a ring system is often incorporated into drugs designed as anticancer,[10,11] antiviral,[12] antihypertensive,[13] analgesic,[14,15] anti-inflammatory,[16] antifungal,[17] antibacterial[17] and anti-psoriasis agents.[18] Aromatic and heteroaromatic compounds bearing an *o*-aminonitrile group are useful substrates for the synthesis of various condensed pyrimidine systems.[19–27] In the present investigation we are reporting a novel building block (the title compound) to give access to the target condensed pyrimidines *via* intramolecular exo-dig annulations.

Results and Discussion

The title compound **2** is prepared by the reaction of a solution of lauroyl isothiocyanate **1** in a dry acetonitrile with 2-amino-4,5,6,7-tetrahydrobenzo[*b*]-thiophene-3-carbonitrile at room temperature (Chart 1). The spectral properties of the new product agree with its proposed structure. Thus its multiple NH groups give rise to bands at 3233 and 3193 cm^{-1} in its infrared (IR) spectrum. Intense broad band at 1531 cm^{-1}, indicative of C–N–H vibration is regarded as combination bands due to NH-deformation and C–N stretching. It shows stretching C–H bands at 2920 and 2849 cm^{-1}. Its carbonyl group produced absorption band at 1695 cm^{-1}. The ^{1}H-NMR spectrum of compound **2** is presented in Experimental and is in accordance with its proposed structure. The higher δ value of NH_a proton at δ 14.22 ppm, suggests the existence of compound **2** as its cheleated form shown in Chart 1. Mass spectrum of compound **2** revealed correct molecular ion peak at $M^{+\cdot}$, (*m*/*z* 419) in addition to some important fragments peaks consistence with its proposed structure.

The idea behind the uses of the title compound **2** as a useful building block for synthesis of annulated heterocyclic systems depends on the fact that reaction of **2** with different nucleophilic reagents (*e.g.*, hydrazines, thiourea, *etc.*) gives intermediates containing nitrile group. The electron deficient carbon of the nitrile site in these intermediates initiated the intramolecular exo-dig annulation to provide the target heterocyclic compounds.

Treatment of a solution of the adduct **2** in ethanol with an equivalent amount of freshly prepared sodium ethoxide at room temperature afforded tetrahydrobenzothieno[2,3-*d*]-1,3-diazine derivative **3** (Chart 2). On the other hand refluxing a solution of **2** in ethanol with NaOH (3 M) produced the amide derivative **4**. Furthermore, boiling of compound **2** in ethanolic hydrochloric acid solution produced tetrahydrobenzothieno[2,3-*d*]-1,3-thiazine derivative **5** in a good yield. The infrared spectra of compounds **3**–**5** showed absorption frequencies correlated with NH group, as well as, C=N group for compounds **3**, **5** and in addition to C=O group for compounds **4**, **5**. Their ^{1}H-NMR spectra displayed the aliphatic protons, beside the acidic NH protons in the downfield

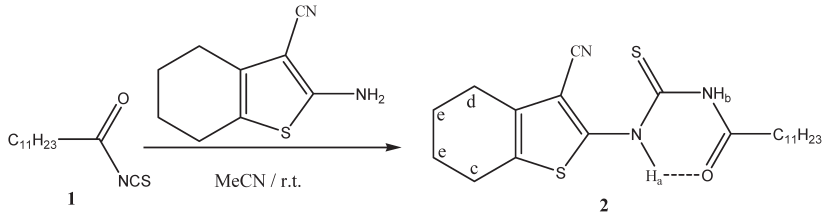

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Chart 1. Reaction of Lauroyl Isothiocyanate with 2-Amino-4,5,6,7-tetrahydrobenzo[*b*]-thiophene-3-carbonitrile

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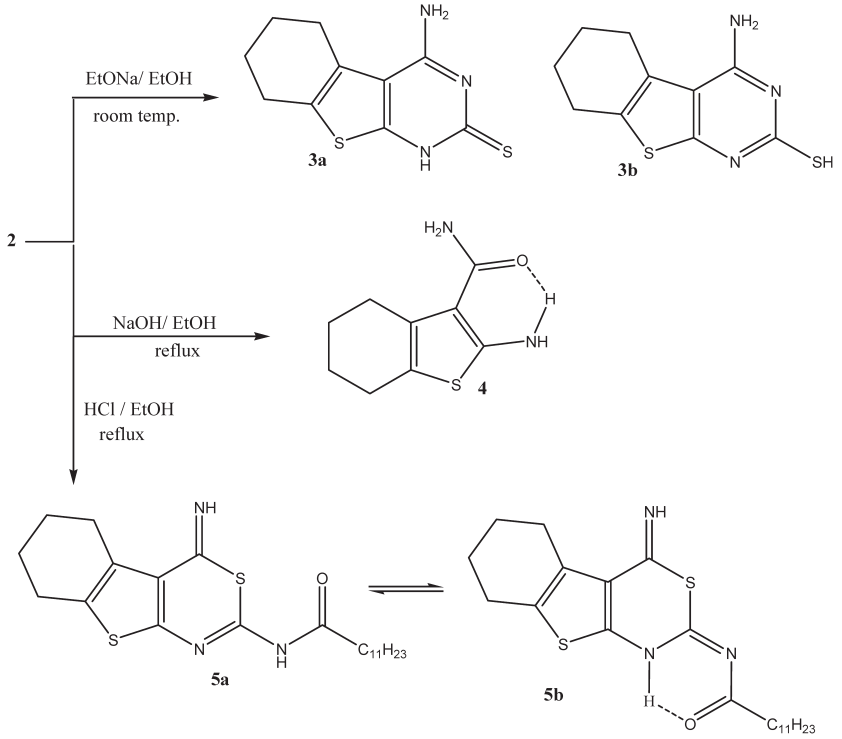

Chart 2. Reaction of the Title Compound with Sodium Ethoxide, Sodium Hydroxide or Hydrochloric Acid in Ethanol

region that was exchangeable with D_2O shake. ^{1}H-NMR spectrum of compound **3** in dimethyl sulfoxide (DMSO) solution, showed its existence in the thiolactim form **3b**. The appearance of the two protons of amide group of compound **4** in the downfield region as two broad singlets is a good evidence for their magnetic non-equivalence. This suggests the existence of compound **4** as its chelated form shown in Chart 2. This is in harmony with the lower frequency of absorption of C=O in its IR spectrum. ^{1}H-NMR spectrum of compound **5** reveals the presence of two types NH proton as two broad singlet signals in the downfield region with equal integration values. This is a good evidence for its existence as an equilibrium mixture of the two tautomers **5a** and **b**. The higher δ value of the amino proton of tautomer **5b** (δ 7.29 ppm) may be attributable to the chelation shown in Chart 2 (see Experimental). The formation of compounds **3** and **5** can be explained on the fact that the nitrile site initiated intramolecular exo-diagonal (exo-dig) cyclization *via* addition of NH_b of compound **2** or sulphur atom of its thiol form to the nitrile group followed by expulsion of the lauroyl part in case of compound **3**. Base-catalyzed hydrolysis of compound **2** takes place at its nitrile group and thiouoredo part leads to the formation of compound **4**.

The reaction of compound **2** with hydrazine hydrate and/or phenylhydrazine led to tricyclic products **6** and **3** respectively, in excellent yields. On other hand a solution of **2** in ethanol with ethyl carbazate in the presence of a catalytic amount of piperidine gave a double annelated product **7** (Chart 3). The microanalytical and spectral data of compounds **6, 7** are presented in Experimental and in agreement with their proposed structures. Their ^{1}H-NMR spectra displayed signals corresponding to aliphatic protons as well as acidic NH and OH protons in the downfield region that was exchangeable with D_2O. Moreover, their mass spectra showed the correct molecular ion peaks and important fragment peaks in agreement with

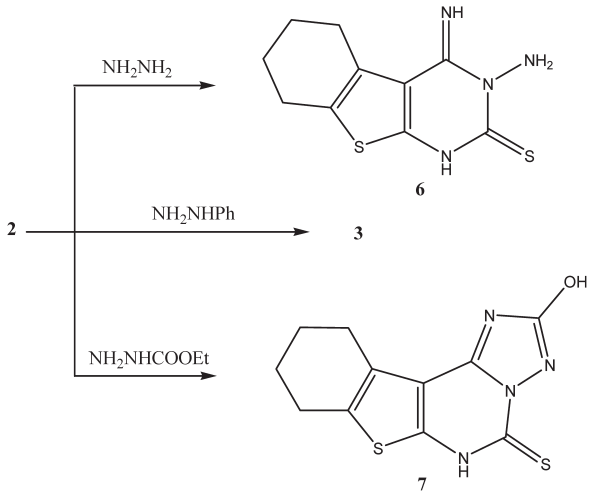

Chart 3. Reaction of the Title Compound with Hydrazine Hydrate, Phenylhydrazine or Ethyl Carbazate in Ethanol

their proposed structures.

The formation of compounds **3**, **6**, **7** can be represented as shown in Chart 4. It is believed that the formation of thiourea derivative **A**, is a common intermediate in these reactions, since it is formed as a result of attack of hydrazine hydrate, phenylhydrazine or ethyl carbazate to the carbonyl group of compound **2**, followed by removal of the lauroyl part in its hydrazide form. The electron deficient carbon of the nitrile site in the thiourea intermediate **A** initiated the intramolecular exo-dig annulation to provide **3** in case of phenylhydrazine or adds another mole of hydrazine or ethyl carbazate to give the other key intermediates which initiated intramolecular exo-dig annulation to provide targets compounds **6** and **7**, respectively.

Refluxing a solution of compound **2** in ethanol with thio-

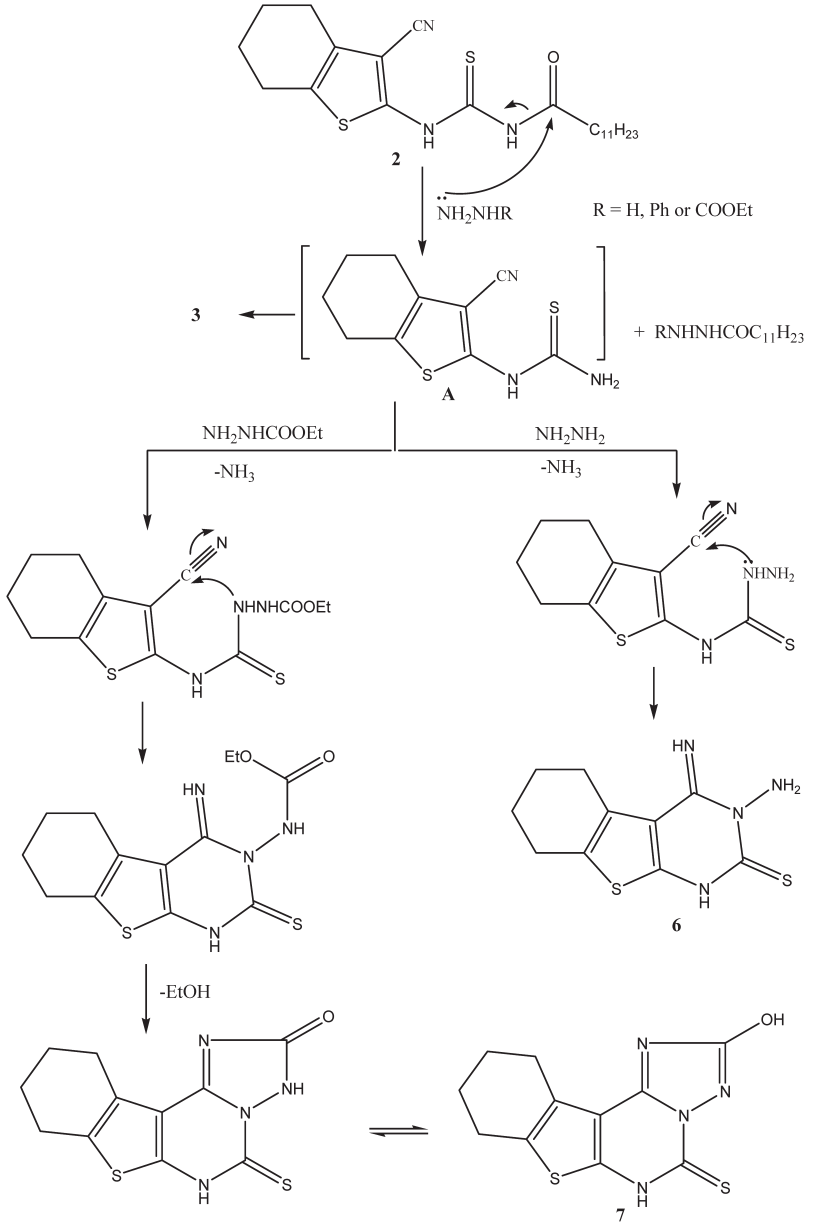

Chart 4. The Proposed Mechanisms of Formation of the Target Products of Reaction of the Title Compound with Hydrazine Hydrate, Phenylhydrazine or Ethyl Carbazate

urea in the presence of a catalytic amount of sodium hydroxide afforded fused tetracyclic compound **8**. On the other hand, the reaction of compound **2** with 2-aminothiophenol in the presence of a catalytic amount of piperidine gave a fused pentacyclic derivative **9** (Chart 5). The appearance of the amino proton (NH) of compound **8** as two broad singlets suggests its existence in DMSO solution as an equilibrium mixture of tautomers **8a** and **b** in ratio of 3 : 1. The product of the reaction of compound **2** with 2-aminothiophenol may have one of the two possible structures **9** or **10**. Structure **9** is more acceptable one on the basis of its ^{1}H-NMR spectrum that displayed two singlet signals equivalent to one proton in the downfield region corresponding to NH and SH proton in the ratio 10 : 1.

The formation of compound **8** can be explained on the basis of cyclocondensation of thiourea with compound **2** to give intermediate in which the nitrile site initiated the intramolecular exo-dig annulations to provide the target heterocyclic system **8** as shown in Chart 6. The formation of compound **9** can be explained as depicted in Chart 7.

Biological Activity The antimicrobial screening of some of the synthesized compounds was done using the hole diffusion method. The possible antimicrobial activities of compounds **2**, **3**, **5**, **7**–**9** were investigated to six standard organisms including the Gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus* and the Gram-negative bacteria, *Escherichia* (*E.*) *coli* and *Pseudomonas*, in addition to unicellular fungi *yeast* and multicellular fungi *Aspergillus flavus*. The obtained results are presented in Table 1. Standard solutions of Septrin D.S. (antibacterial agent) and Fungistatin (antifungal agent) were served as positive controls.

Data in Table 1 emphasized the fact that the chemical agent symbolized **2** is exhibited low activity only against *yeast* (the unicellular fungi), compound **3** revealed low activity only against *Bacillus subtilis* (Gram-positive bacteria) and the

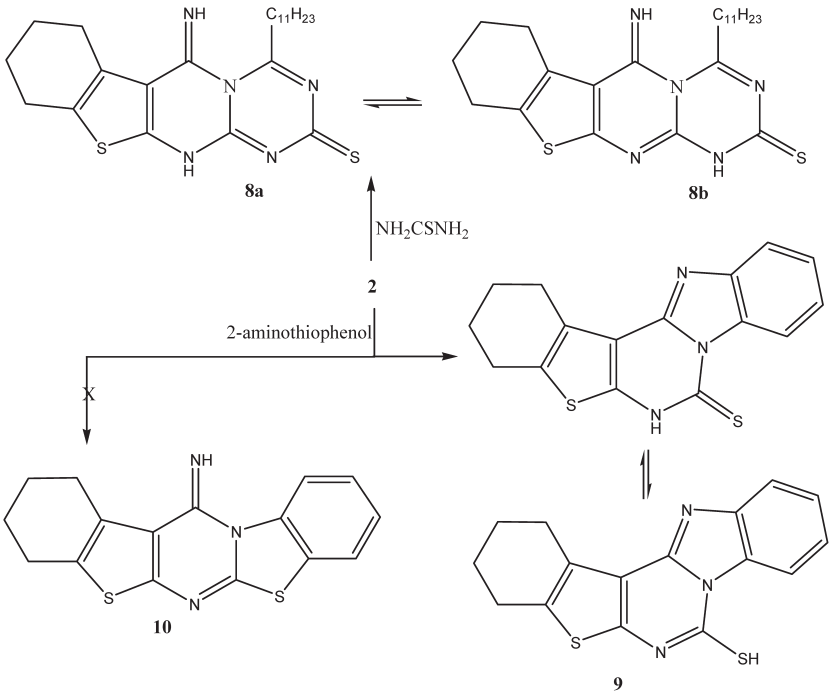

Chart 5. Reaction of the Title Compound with Thiourea, and 2-Aminothiophenol in Ethanol

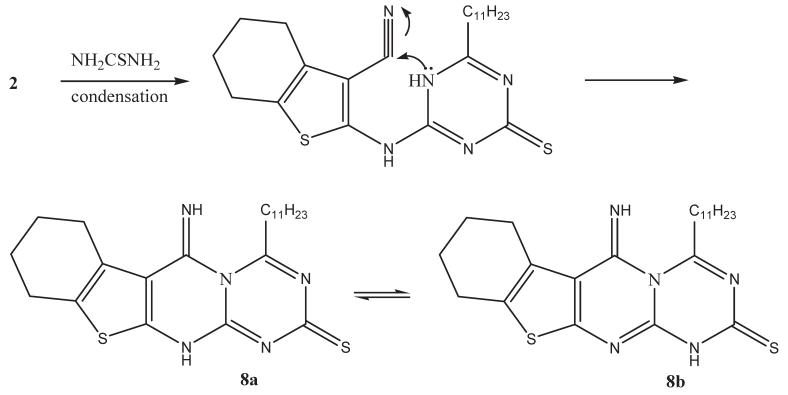

Chart 6. The Proposed Mechanism of Formation of the Target Product of Reaction of the Title Compound with Thiourea

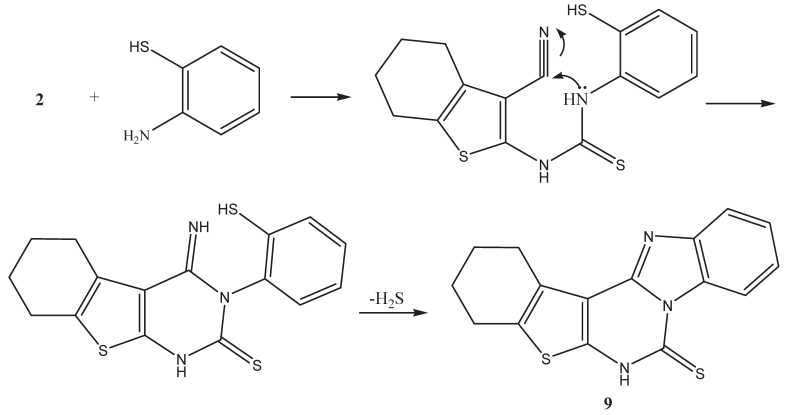

Chart 7. The Proposed Mechanism of Formation of the Target Product of Reaction of the Title Compound with 2-Aminothiophenol

chemical agent **9** showed low activity against both *Staphylococcus aureus* (Gram-positive bacteria) and *Pseudomonas* (Gram-negaitive bacteria) and didn't show activity against the other microorganisms. The other compounds **5**, **7** and **8** didn't show activity at all against the examined microorganisms.

Table 1. Antimicrobial Activity of Selected Compounds

No.	Inhibition zone diameter (cm/gm sample)					
	Bacillus subtilis G^+	*Staphylococcus aureus* G^+	*E. coli* G^-	*Pseudomonas* G^-	Yeast (unicellular fungi)	*Aspergillus flavus* (multicellular fungi)
2	—	—	—	—	5	—
3	5	—	—	—	—	—
5	—	—	—	—	—	—
7	—	—	—	—	—	—
8	—	—	—	—	—	—
9	—	5	—	5	—	—
S	25	30	40	25		
F					20	15

S=Septrin D.S. (antibacterial agent), **F**=Fungistatin (antifungal agent). The concentration of all synthesized compounds and the reference was (5 mg/1 mL of DMSO). Zone of inhibition: <15 mm (low); 15–24 mm (moderate); 25–34 mm (high); 35–44 mm (very high); —: no inhibition.

Experimental

Melting points of the reaction products were determined in open capillary tubes on an electrothermal melting point apparatus and were uncorrected. The elemental analyses were performed on a Perkin-Elemer 2400 CHN elemental analyzer. The infrared spectra were recorded on Perkin-Elemer Modle 297 Infrared spectrometer using the KBr wafer technique. The ^{1}H-NMR spectra were measured on Varian Gemini 300 MHz spectrometer, with chemical shift (δ) expressed in ppm downfield with tetramethylsilane (TMS) as internal standard, in DMSO-d_6. Mass spectra were determined on Shimadzu GC-MSQP 1000 EX instrument operating at 70 eV. Thin layer chromatography (TLC) was run using TLC aluminum sheets silica gel F_{254} (Merck). It was carried out the monitoring of the progress of all reactions and homogeneity of the synthesized compounds.

1-(3-Cyano-4,5,6,7-tetrahydrobenzo[*b*]thiophen-2-yl)-3-dodecanoylthiourea (2) A solution of lauroyl isothiocyanate (3 mmol) and 2-amino-4,5,6,7-tetrahydrobenzo[*b*]-thiophene-3-carbonitrile (3 mmol) in 30 mL dry acetonitrile was stirred at room temperature for 2 h. The resulting yellow solid product was collected by filtration and recrystallized from ethanol to give **2** as pale yellow crystals. Yield 86%; mp 132–134°C; ^{1}H-NMR (DMSO-d_6) δ: 0.84 (3H, $\underline{CH_3}(CH_2)_8CH_2CH_2CO$, t, J=6.6 Hz), 1.24 (16H, $CH_3(\underline{CH_2})_8CH_2CH_2CO$, m), 1.56 (2H, $CH_3(CH_2)_8\underline{CH_2}CH_2CO$, m), 1.76 (4H$_e$, 2CH$_2$, m), 2.44 (2H, $CH_3(CH_2)_8CH_2\underline{CH_2}CO$, t, J=7.5 Hz), 2.54 (2H$_d$, CH$_2$, m), 2.62 (2H$_c$, CH$_2$, m), 11.97 (1H, NH$_b$, exchangeable, brs), 14.22 (1H, NH$_a$, exchangeable, brs); IR (KBr) ν: 3233, 3193 (NH), 2920, 2849 (C–H$_{alkyl}$), 2211 (CN), 1695 (C=O$_{amide}$), 1588 (C=C), 1200 (C=S); MS (70 eV) m/z (%): 419 ($M^{+\cdot}$, 30), 360 (3), 237 (27), 220 (100), 204 (3), 192 (72), 178 (88), 160 (4), 150 (22), 134 (7), 59 (37). *Anal.* Calcd for $C_{22}H_{33}N_3OS_2$ (419.65): C, 62.97; H, 7.93; N, 10.01. Found C, 62.82; H, 7.87; N, 9.79%.

Uses of Compound 2 in Synthesis Different Target Heterocyclic Systems 4-Amino-5,6,7,8-tetrahydrobenzothieno[2,3-*d*]-1,3-diazine-2-thione (**3**) A mixture of **2** (3 mmol) and sodium ethoxide (3 mmol) in 30 mL absolute ethanol was stirred at room temperature for 4 h. A yellow solid product was obtained, filtered off, and recrystallized from ethanol to give compound **3** yellow crystals. The same product was obtained on refluxing of a solution of **2** (3 mmol) in ethanol (30 mL) and phenylhydrazine (3 mmol) for 4 h. Yield 79%; mp>300°C; ^{1}H-NMR (DMSO-d_6) δ: 1.78 (4H$_e$, 2CH$_2$, m), 2.60 (2H$_d$, CH$_2$, m), 2.70 (2H$_c$, CH$_2$, m), 4.31 (1H, SH, exchangeable, brs), 6.99 (2H, NH$_2$, exchangeable, brs); IR (KBr) ν: 3444, 3340, 3194 (NH), 2933, 2854 (C–H$_{alkyl}$), 1597 (C=N), 1554 (C=C), 1235 (C=S); MS (70 eV) m/z (%): 237 ($M^{+\cdot}$, 57), 219 (16), 204 (17), 179 (39), 150 (30), 121 (18), 71 (56), 57 (100). *Anal.* Calcd for $C_{10}H_{11}N_3S_2$ (237.34): C, 50.60; H, 4.67; N, 17.70. Found C, 50.44; H, 4.65; N, 17.46%.

2-Amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxamide (**4**) A solution of compound **2** (3 mmol) and (3 M) sodium hydroxide (5 mL) in 30 mL ethanol was refluxed for 2 h. Vacuum-distilled to *ca.* half-volume and acidified with 3 M hydrochloric acid. The precipitate was collected and recrystallized from ethanol to give compound **4** as pale yellow crystals; yield 73%; mp>300°C; ^{1}H-NMR (DMSO-d_6) δ: 1.77 (4H$_e$, 2CH$_2$, m), 2.64 (2H$_d$, CH$_2$, m), 2.76 (2H$_c$, CH$_2$, m), 7.19 (2H, NH$_2$, exchangeable, brs), 11.94, 13.03 (2H, CONH$_2$, exchangeable, two brs); IR (KBr) ν: 3440, 3358 (NH$_2$), 3220, 3176 (NH), 2933, 2840 (C–H$_{alkyl}$), 1632 (C=O), 1553 (C=C); MS (70 eV) m/z (%): 196 ($M^{+\cdot}$, 4), 185 (6), 179 (50), 178 (67), 163 (16), 150 (100), 134 (25), 116 (19). *Anal.* Calcd for $C_9H_{12}N_2OS$ (196.27): C, 55.08; H, 6.16; N, 14.27. Found C, 54.87; H, 5.81; N, 13.96%.

N-(4-Imino-5,6,7,8-tetrahydrobenzothieno[2,3-*d*][3,1]thiazin-2-yl)dodecanamide (**5**) A solution of **2** (3 mmol) in 30 mL ethanol and 3 M hydrochloric acid (5 mL) was refluxed for 3 h. The solution was vacuum-evaporated to small volume. A solution of sodium carbonate (0.1 N) was added until effervescence ceased. The yellow precipitate was filtered off and recrystallized from ethanol to give compound **5** as pale yellow crystals; yield 81%; mp 196–198°C; ^{1}H-NMR (DMSO-d_6) δ: 0.85 (3H, $\underline{CH_3}(CH_2)_8CH_2CH_2CO$, t, J=6.6 Hz), 1.30 (16H, $CH_3(\underline{CH_2})_8CH_2CH_2CO$, m), 1.54 (2H, $CH_3(CH_2)_8\underline{CH_2}CH_2CO$, m), 1.73 (4H$_e$, 2CH$_2$, m), 2.40 (2H, $CH_3(CH_2)_8CH_2\underline{CH_2}CO$, m), 2.68 (4H, 2CH$_2$, m), 11.38 (1H, NH=, exchangeable, brs), for **5a**: 14.08 (1H, NHCO, exchangeable, brs), for **5b**: 7.29 (1H, NH, thiazine, exchangeable, brs); IR (KBr) ν: 3411, 3322, 3195 (NH), 2920, 2850 (C–H$_{alkyl}$), 1704 (C=O), 1633, 1592 (C=N), 1559, 1531 (C=C MS (70 eV) m/z (%): 419 ($M^{+\cdot}$, 3), 403 (25), 368 (1), 263 (2), 221 (100), 193 (29), 178 (8), 150 (3). *Anal.* Calcd for $C_{22}H_{33}N_3OS_2$ (419.65): C, 62.97; H, 7.93; N, 10.01. Found C, 63.14; H, 7.72; N, 9.68%.

Reaction of Compound 2 with the Nitrogen Nucleophiles General Procedure To a solution of compound **2** (3 mmol) in ethanol (30 mL), hydrazine hydrate (3 mmol) was added. The reaction mixture was refluxed for 4 h, then, cooled to

room temperature. A solid was obtained that was filtered off and recrystallized from ethanol to give compound **6**. The same procedure was done with ethyl carbazate, thiourea and/or *o*-aminothiophenol. A catalytic amount of piperidine was added in case of reaction with ethyl carbazate and *o*-aminothiophenol while, a catalytic amount of sodium hydroxide was added in case the reaction of **2** with thiourea. The progress of all reactions and homogeneity of the synthesized compounds were monitored by TLC. The solid obtained for each case was recrystalized from a suitable solvent to give the corresponding compound.

3-Amino-4-imino-2-thioxo-5,6,7,8-tetrahydrobenzothieno[2,3-*d*]-1,3-diazine (**6**) Pale yellow crystals; yield 85%; mp 271–273°C (EtOH); ^{1}H-NMR (DMSO-d_6) δ: 1.77 ($4H_e$, $2CH_2$, m), 2.63 ($2H_d$, CH_2, m), 2.76 ($2H_c$, CH_2, m), 7.93 (2H, NH_2, exchangeable, brs), 8.35 (1H, NH, exchangeable, brs), 10.22 (1H, NH=, exchangeable, brs); IR (KBr) ν: 3428, 3310, 3250, 3120 (NH), 2925, 2829 (C–H_{alkyl}), 1630 (C=N), 1573, 1533 (C=C), 1186 (C=S); MS (70 eV) *m/z* (%): 252 ($M^{+\cdot}$, 9), 237 (100), 221 (12), 204 (10), 192 (5), 179 (34), 160 (7), 134 (6), 118 (8). *Anal.* Calcd for $C_{10}H_{12}N_4S_2$ (252.36): C, 47.59; H, 4.79; N, 22.20. Found C, 47.43; H, 4.46; N, 21.87%.

2-Hydroxy-8,9,10,11-tetrahydrobenzothieno[2′,3′ : 4,5]pyrimido-[3,4-*b*]-1,2,4-triazole-5(6*H*)-thione (**7**) Pale brown crystals; yield 79%; mp>300°C (EtOH); ^{1}H-NMR (DMSO-d_6) δ: 1.78 ($4H_e$, $2CH_2$, m), 2.69 ($2H_d$, CH_2, m), 2.85 ($2H_c$, CH_2, m), 7.02 (2H, NH and OH, exchangeable, brs); IR (KBr) ν: 3448 (OH), 3306, 3208 (NH), 2924, 2839 (C–H_{alkyl}), 1625 (C=N), 1547 (C=C), 1229 (C=S); MS (70 eV) *m/z* (%): 278 ($M^{+\cdot}$, 38), 272 (43), 261 (40), 248 (36), 231 (45), 218 (48), 194 (45), 141 (12), 129 (34), 69 (100). *Anal.* Calcd for $C_{11}H_{10}N_4OS_2$ (278.35): C, 47.46; H, 3.62; N, 20.13. Found C, 47.18; H, 3.71; N, 19.76%.

6-Imino-4-undecyl-6,12-dihydro-2*H*-7,8,9,10-tetrahydrobenzothieno[2′,3′ : 4,5]pyrimido[1,2-*a*][1,3,5]triazine-2-thione (**8**) Colorless crystals; yield 81%; mp 251–253°C (EtOH); ^{1}H-NMR (DMSO-d_6) δ: 0.85 (3H, $\underline{CH_3}(CH_2)_8CH_2CH_2CO$, t, J=6.9 Hz), 1.24 (16H, $CH_3(\underline{CH_2})_8CH_2CH_2CO$, m), 1.47 (2H, $CH_3(CH_2)_8\underline{CH_2}CH_2CO$, m), 1.81 ($4H_e$, $2CH_2$, m), 2.17 (2H, $CH_3(CH_2)_8CH_2\underline{CH_2}CO$, t, J=7.5 Hz), 2.79 ($2H_d$, CH_2, m), 2.91 ($2H_c$, CH_2, m), 7.47 (1H, NH=, exchangeable, brs), for **8a**: 6.80 (1H, NH, exchangeable, brs), for **8b**: 6.94 (1H, NH, exchangeable, brs); IR (KBr) ν: 3442, 3294, 3113 (NH), 2934, 2856 (C–H_{alkyl}), 1648 (C=N), 1565 (C=C), 1272 (C=S); MS (70 eV) *m/z* (%): 443 ($M^{+\cdot}$, 80), 417 (65), 381 (50), 368 (81), 331 (72), 313 (88), 260 (48), 224 (98), 205 (94), 155 (94), 106 (100), 81 (91). *Anal.* Calcd for $C_{23}H_{33}N_5S_2$ (443.67): C, 62.26; H, 7.50; N, 15.78. Found C, 61.89; H, 7.22; N, 15.64%.

10,11,12,13-Tetrahydrobenzothieno[2′,3′ : 4,5]pyrimido[3,4-*a*]-benzimidazol-7(8*H*)-thione (**9**) Brown crystals; yield 68%; mp>300°C (EtOH); ^{1}H-NMR (DMSO-d_6) δ: 1.78 ($4H_e$, $2CH_2$, m), 2.69 ($2H_d$, CH_2, m), 2.85 ($2H_c$, CH_2, m), 6.10 (1H, SH, exchangeable, brs), 6.46 (1H, Ar-H, t), 6.66 (1H, d, J=8.1 Hz), 7.01 (1H, NH, exchangeable, brs), 7.07 (1H, Ar-H, t), 7.41 (1H, d, J=7.5 Hz); IR (KBr) ν: 3444, 3342, 3192 (NH), 2933, 2855 (C–H_{alkyl}), 1597 (C=N), 1545 (C=C), 1233 (C=S); MS (70 eV) *m/z* (%): 311 ($M^{+\cdot}$, 34), 287 (21), 243 (30), 208 (23), 180 (26), 151 (31), 119 (3), 77 (9), 66 (100). *Anal.* Calcd for $C_{16}H_{13}N_3S_2$ (311.42): C, 61.71; H, 4.21; N, 13.49. Found C, 61.60; H, 4.14; N, 13.21%.

Measurement of Antimicrobial Activity Using the Hole Diffusion Method Dissolve the samples in DMSO (5 mg/1 mL). Make bacterial suspension and fungal suspension in Nutrant broth media for bacteria and Sabarude broth media for Fungi. Take 1 mL of bacterial or fungal suspension in sterallized petri dishes and pour media on it and mix well to homogenized inoculums with media. After media solidified, make holes in media with courk pourer. Add 50 μg of samples solution and control solution in holes. Incubate plates at 37°C for 24 h. Investigate plate after incubation period by measure diameter of inhibition zone and get courk pourer diameter. Actually inhibition zone=D. of inhibition zone−D. of courk pourer.

Conflict of Interest The authors declare no conflict of interest.

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