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Original article

Thieno[2,3-*d*]pyrimidinedione derivatives as antibacterial agents

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ABSTRACT

Several thieno[2,3-*d*]pyrimidinediones have been synthesized and examined for antibacterial activity against a range of Gram-positive and Gram-negative pathogens. Two compounds displayed potent activity (2–16 mg/L) against multi-drug resistant Gram-positive organisms, including methicillin resistant, vancomycin-intermediate, vancomycin-resistant *Staphylococcus aureus* (MRSA, VISA, VRSA) and vancomycin-resistant enterococci (VRE). Only one of these agents possessed moderate activity (16–32 mg/L) against Gram-negative strains. An examination of the cytotoxicity of these agents revealed that they displayed low toxicity (40–50 mg/L) against mammalian cells and very low hemolytic activity (2–7%). Taken together, these studies suggest that thieno[2,3-*d*]pyrimidinediones are interesting scaffolds for the development of novel Gram-positive antibacterial agents.

1. Introduction

The increasing prevalence of pathogenic bacteria that are resistant to currently available antibiotics represents an alarming threat to public health. The most commonly encountered antibiotic-resistant bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA), has had a major impact on infections in both the hospital and community setting [1,2]. While vancomycin continues to be the standard treatment option for antibiotic-resistant infections, the isolation of vancomycin-resistant *Staphylococcus* (VRSA) and *Enterococci* (VRE) foreshadows a day in which the utilization of vancomycin may become limited [3]. Unfortunately, as antibiotic-resistant organisms have become more commonplace, the pipeline for the discovery of new antimicrobial agents has decreased [4]. Thus, there is a pressing need for new antimicrobial agents that are capable of treating resistant bacterial strains.

Thienopyrimidines are interesting heterocyclic compounds and a number of derivatives of these compounds display therapeutic activity as antimicrobial [5–7], antiviral [8,9], antiinflammatory [10] antidiabetic [11] and anticancer [12,13] agents [14–16]. Despite the breadth of biological activities displayed by these agents, the antibacterial activity of this class of compounds has been underexplored. El-Sherbeny and colleagues examined the antimicrobial and antiviral activity of cyclopenteno and cyclohexeno [*b*]thieno[2,3-*d*]-3,4-dihydropyrimidine-4-one derivatives (Fig. 1a) [8]. These agents displayed reasonable activity (MIC values 6.25–25 mg/L) against both Gram-positive and Gram-negative bacteria; however, these agents were significantly more potent against herpes simplex virus [8]. Furanyl-thieno[2,3-*d*]pyrimidin-4-ones (Fig. 1b) were examined by Bahekar et al. for their antibacterial activity [7]. These agents displayed MIC values in the range of 4–100 mg/L against a collection of Gram-positive and Gram-negative microbes. Interestingly, these compounds also displayed antimycobaterial activity [7]. The antibacterial activity of thieno[2,3-*d*]pyrimidindiones (Fig. 1c) has not been reported in the literature; however, these compounds have been examined for antiviral activity [15].

Recently, during a study on thieno[2,3-*d*]pyrimidinones, we discovered a set of compounds that possessed antibacterial activity (Fig. 1d). These agents are structurally unrelated to any clinically used antibiotic and display discreet structural overlap with thieno [2,3-*d*]pyrimidines that have been reported in the literature. In this report, we discuss the synthesis of thieno[2,3-*d*]pyrimidinediones and their antibacterial and cytotoxic activities.

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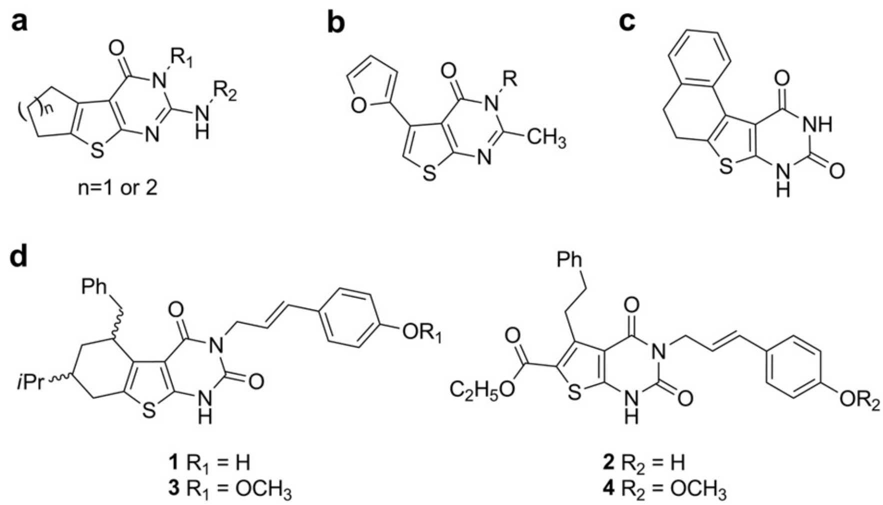


Fig. 1. Thieno[2,3-*d*]pyrimidineone derivatives.

2. Results and discussion

2.1. Chemistry

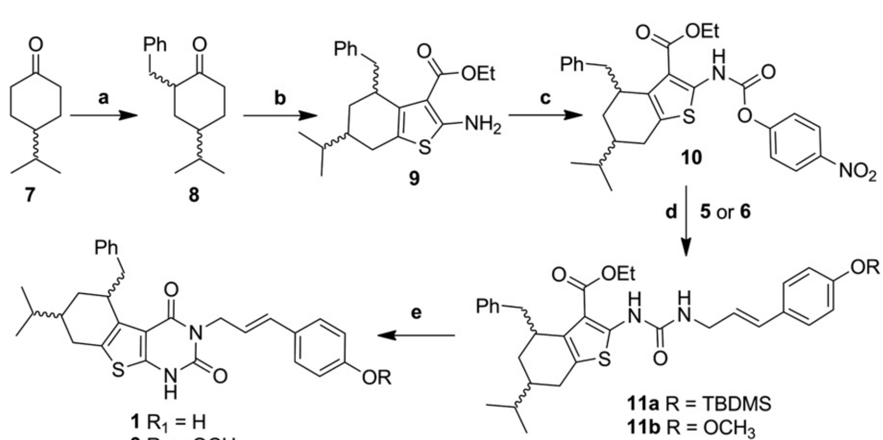
For a project on the development of antiviral therapeutics, we required the synthesis of two constrained (**1** and **3**) and two unconstrained (**2** and **4**) thieno[2,3-*d*]pyrimidine-2,4-dione derivatives (Fig. 1d), neither of which had been described in the literature. A retrosynthetic analysis of these agents suggested that an amino thiophene ester ring would be prepared first using the standard Gewald reaction [17,18]. Once the thiophene was in hand, the pyrimidine ring could be prepared by converting the amine into a urea followed by cyclizing with the ester under basic conditions.

The synthesis of compounds **1**–**4** is shown in schemes 1–3. The synthesis of the constrained derivatives **1** and **3** starts from the commercially available ethyl-2-cyanoacetate and racemic benzylated isopropyl cyclohexanone (**8**, prepared from **7**). These were reacted with sulfur to obtain the amino thiophene ester, **9**, in decent yields. Activation of **9** with *p*-nitrophenyl chloroformate generated the unstable intermediate **10**, which upon reaction with amines **5** or **6** produced urido compounds **11a** and **11b** [19]. Formation of the pyrimidinedione ring was accomplished with refluxing sodium methoxide to provide the final compounds **1** and **3** (Scheme 1) [16]. The unconstrained compounds **2** and **4** were prepared using similar methodology starting from the ethyl 3-oxo-5-phenylpentanoate, **13** (Scheme 2).

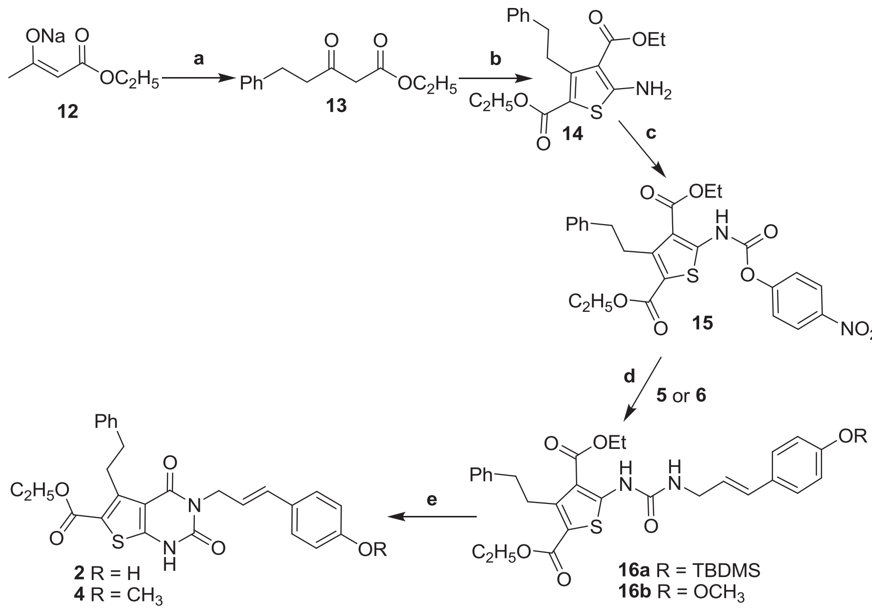
The amines, **5** and **6**, were prepared, as shown in the Scheme 3, from commercially available compounds (**17** and **21**) using procedures published for related compounds [20–26]. Amine **5** was prepared from *p*-hydroxycinnamic acid (**17**) by first protecting the phenol as the silyl ether followed by conversion of the acid into the azide (**20**). Selective reduction of the azide to **5** was accomplished using catalytic hydrogenation in the presence of Lindlar's catalyst ($Pd/CaCO_3$). Amine **6** was synthesized from *p*-iodoanisole (**21**) using a Heck reaction with acrylonitrile to generate *E*-cinnamonitrile (**22**). Reduction of the nitrile to the amine was accomplished with $LiAlH_4$.

2.2. In vitro antibacterial activity

The initial examination of compounds **1**–**4** failed to detect antiviral activity. As part of a further investigation of the biological activities of these compounds, we examined the antibacterial activity of compounds **1**–**4** and intermediates **11a**, **11b**, **16a** and **16b** against a panel of five Gram-positive and four Gram-negative bacteria (Table 1). Compounds **1** and **2** demonstrated significant

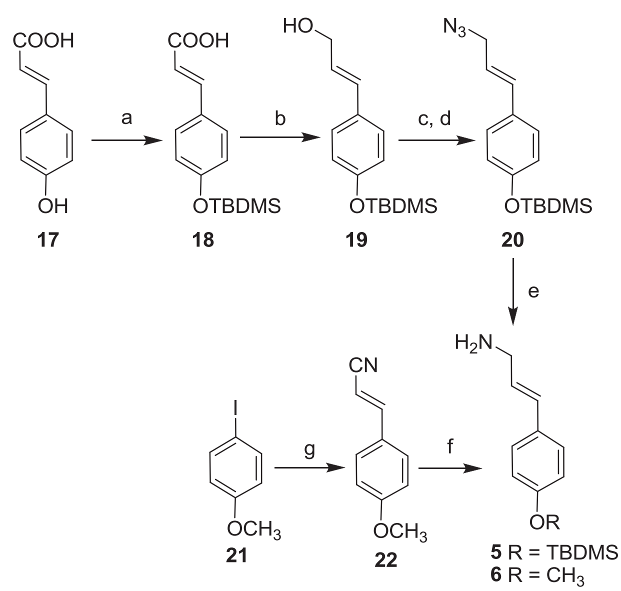


Scheme 1. a) *n*BuLi, BnBr, THF, −78 °C to r.t.,18 h, 80% b) ethyl 2-cyanoacetate, S_8, morpholine, EtOH, Δ, 24–48 h., 39% c) *p*-nitrophenyl chloroformate, pyridine, CH_2Cl_2, r.t. 6–12 h, 77% d) pyridine, DMAP, THF, r.t. 12–24 h, 69–79% e) NaOMe, MeOH, reflux, 3 h, 76–77%.



Scheme 2. a) *n*BuLi, BnBr, THF, 0–30 °C,18 h, 66% b) ethyl 2-cyanoacetate, S_8, morpholine, EtOH, Δ, 24–48 h, 38% c) *p*-nitrophenyl chloroformate, pyridine, CH_2Cl_2, r.t. 6–12 h, 77% d) pyridine, DMAP, THF, r.t. 12–24 h, 80–85% e) NaOMe, MeOH, reflux, 3 h, 88–90%.

antibacterial activity with MIC values in the 2–16 mg/L range against Gram-positive bacteria such as MRSA, VRSA, VISA, VRE and *Streptococcus pneumoniae*. The Gram-negative activity of **1** and **2** was weak with MIC values in the range of 16 to over 32 mg/L. Compounds **3** and **4** displayed almost no antibacterial activity with the exception being the moderate activity (8 mg/L) **3** displayed against *Enterobacter aerogenes*. Compounds **11a**, **16a** and **16b** showed no activity against any bacterial strain; compound **11b** was insoluble in media, water and DMSO and thus could not be tested. Taken together, the data support the conclusion that **1** and **2** possess Gram-positive antibacterial activity, while compounds **3**, **4**, **11a**, **16a** and **16b** are essentially inactive.



Scheme 3. a) TBDMSCl, imidazole/DMF, r.t. 3 h, 98% b) DIBAL/CH_2Cl_2, −78 °C, 8 h, 59% c) MsCl, Et_3N/THF d) NaN_3/DMF, 82% e) Pd/CaCO3, H_2(g)/EtOH, r.t.,93% f) $LiAlH_4$/Et_2O, r.t. 15 min, 61% g) Acrylonitrile, $Pd(OAc)_2$, Bu_4NBr, $NaHCO_3$/H_2O, 48%.

2.3. *In vitro cytotoxicity*

We examined the cytotoxic activity of compounds **1**–**4** against mammalian cells (NIH-3T3) using the MTS assay. As shown in Fig. 2, all compounds displayed moderate levels of toxicity at 100 mg/L (<50% of cell viability). The LD_{50} of each compound was determined from a dose–response curve of cytotoxicity versus concentration (Table 1). As shown in the table, the LD_{50} (∼52 mg/L) of the most potent compound, **2**, is 25 times higher than its MIC value indicating that **2** is selectively toxic to bacteria. In contrast, **1** displayed a therapeutic index of only 5 suggesting that the toxicity of the compound may have also played a role in its antibacterial activity.

2.4. *In vitro hemolytic activity*

Another common measure of the toxicity of an agent is lysis of red blood cells. We examined the ability of compounds **1** and **2** to lyse Sheep erythrocytes. As shown in Fig. 3, compound **1** displayed approximately 7% hemolysis at a concentration 25 times its MIC value. In contrast, **2**, which is the most selective antibacterial agent described here, showed only 2% hemolysis at 50 times its MIC value. These results indicate that the antibacterial activity observed for compounds **1** and **2** is not due to non-specific membrane damage. This would suggest that there are specific antibacterial targets for these agents in bacteria.

2.5. *Discussion*

In this report we have explored thieno[2,3-*d*]pyrimidinedione derivatives as antibacterial agents. While thieno[2,3-*d*]pyrimidines have been extensively explored for their varied biological activity, the compounds reported here are unique and have not been prepared in the literature. Among the compounds synthesized, compound **2** showed selective antibacterial activity against Gram-positive antibiotic-resistant bacterial strains such as MRSA, VRSA, VISA and VRE. Unfortunately, these compounds were inactive against Gram-negative pathogens.

Table 1
MIC and LD_{50} values for compounds **1**–**4**, **11a**, **11b**, **16a**, and **16b**.

	MIC (mg/L)									LD_{50} (mg/L)	Therapeutic Index[a]
	S. aureus (VISA Mu50)	*S. aureus* (MRSA 494)	*S. aureus* (VRSA MI)	*S. pneumoniae* (ATCC 49619)	*E. faecium* (VRE 7303)	*E. coli* (ATCC 25922)	*P. aeruginosa* (ATCC 27853)	*K. pneumoniae* (CEF 2324)	*E. aerogenes* (CEF 3978)		
1	16	16	8	32	8	32	16	32	32	42	5
2	2	2	2	8	4	>32	32	32	16	52	26
3	>32	>32	>32	32	>32	>32	>32	>32	32	120	4
4	>32	>32	>32	>32	>32	>32	>32	>32	8	98	3
11a	>32	>32	>32	>32	>32	>32	>32	>32	>32	nd	nd
11b[b]	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
16a	>32	>32	>32	>32	>32	>32	>32	>32	>32	nd	nd
16b	>32	>32	>32	>32	>32	>32	>32	>32	>32	nd	nd
Vancomycin	nd	0.5	nd	0.5	>32	nd	>32	nd	nd	nd	nd

[a] Therapeutic index was calculated as the ratio of LD_{50}/MIC for VRSA.
[b] Not determined due to insolubility of compound in media.

A complete structure–activity relationship cannot be determined from the limited set of compounds prepared here. However, inactivity of urido compounds (**11a**, **16a** and **16b**) clearly demonstrates the importance of the pyrimidine ring for the activity and our data also suggests that the presence of a phenol is critical for activity (compare compound **2** to compound **4**). Furthermore, the presence of a methoxy group increases the cytotoxicity by 2-fold when compared to the phenol. A well-known method for enhancing potency of compounds is to lock flexible portions of the molecule into a fixed conformation. Tethering the side chains located on the thiophene results in the racemic compound **1**. While **1** displayed antibacterial activity against Gram-positive pathogens, the MIC values were 2–8-fold higher for the unconstrained analog **2**. Thus, the flexibility seen in compound **2** appear to be necessary for the compound to adopt a biologically active conformation.

The serendipitous discovery of antibacterial activity for these agents unfortunately means that the mechanism of action is unknown. While there have been reports of antibacterial activity for structurally unrelated thieno-pyrimidinones (see Introduction), the mechanism of action of those agents has never been reported. Thieno-pyrimidines and thieno-pyrimidinediones have been reported as inhibitors of proteases [27], kinases [28–30] and folate utilizing enzymes [8,9,31] suggesting that the mechanism of action of our agent could be due to inhibition of a specific protein. The inhibition of folate utilizing enzymes (i.e. dihydrofolate reductase, etc.) is especially interesting given the fact that antibacterial agents specifically targeting bacterial folate enzymes are well known. Of course, numerous other pathways could be responsible for the antibacterial activity of the compounds, including non-specific membrane effects. We are currently investigating the mechanism of action of these thieno[2,3-*d*]pyrimidinediones and hope to report on their mechanism in due course.

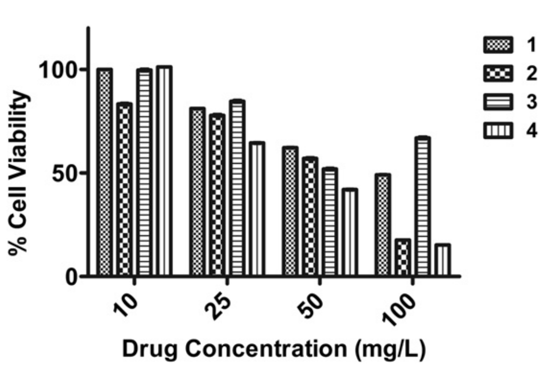


Fig. 2. Cytotoxicity of compounds **1**–**4** obtained my MTS assay against mammalian cells (NIH-3T3), 5% DMSO is used as control.

3. Conclusion

We have synthesized constrained and unconstrained thieno [2,3-*d*]pyrimidinedione derivatives and examined their antibacterial, cytotoxicity and hemolytic activity. Only one compound, **2**, displayed potent antibacterial activity against a wide range of antibiotic-resistant bacteria including MRSA, VRSA, VISA and VRE. This compound was minimally cytotoxic against mammalian cells and possessed essentially no hemolytic activity. While the mechanism of action of this compound is unknown, efforts to determine the reason for its antibacterial activity are ongoing and will be reported in due course.

4. Experimental

4.1. Materials and instruments

All chemicals were purchased from Acros, Sigma–Aldrich, Matrix Scientific, EMD or Frinton labs and used without further purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on Varian DRX400. The mass spectra of respective final compounds were recorded on Waters-Micromass ZQ quadupole located in the central instrumentation facility at Wayne State University, Detroit, MI. For the MTS and hemolysis assays, absorbance was collected

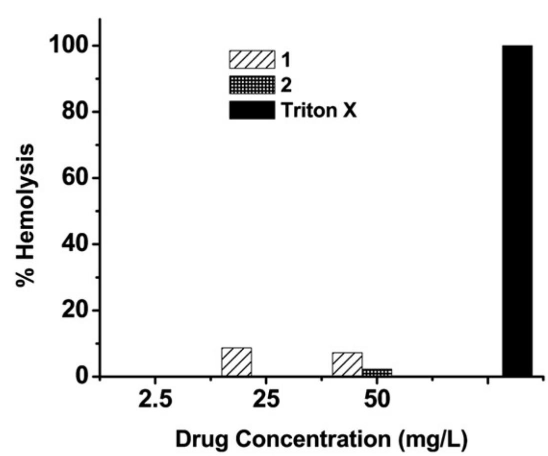


Fig. 3. Hemolytic activity of compounds **1** and **2** against Sheep erythrocytes. Hemolysis was calculated using 1% Triton-X as positive control which set to 100% lysis.

using Synergy 2 multi-mode plate reader from Biotek Instruments Inc. Winooski, VT.

*4.1.1. 2-Benzyl-4-isopropyl cyclohexanone (**8**)*

To a cooled (−20 °C) solution of diisopropylamine (0.160 g, 1.57 mmols) in dry toluene, *n*-butyl lithium (1.6 M in hexanes, 0.95 mL, 1.5 mmols) was added and stirred for 15 min. To the solution, isopropyl cyclohexanone (**7**) (0.200 g, 1.43 mmols), dissolved in 5 mL of dry toluene, was added drop wise. The reaction was stirred at −20 °C for 30 min and then cooled to −78 °C. To the chilled solution, benzyl bromide (0.270 g, 1.57 mmols), dissolved in 5 mL dry toluene, was added drop wise and the reaction mixture was warmed to −45 °C and stirred at this temperature for 18 h. The reaction was quenched with 0.5 N HCl (10 mL) and extracted with ether (150 mL). The combined organic layers were washed with brine, H_2O and then dried over anhydrous Na_2SO_4 before being concentrated *in vacuo*. The crude product was purified by flash silica column chromatography (1:9 ethyl acetate:hexanes) to yield pure **8** (0.26, 80%). ^{1}H NMR (400 MHz, CD_2Cl_2) δ 0.85 (d, J = 2.8 Hz, 3H, CH_3), 0.86 (d, J = 2.4 Hz, 3H, CH_3), 1.44–1.59 (m, 4H, CH_2, CH_2), 2.31–2.37 (m, 4H, CH_2, CH_2), 2.38–2.65 (m, 2H, CH, CH), 3.2 (dd, J_a = 14 Hz, J_b = 5.2 Hz, 1H, CH), 7.15–7.28 (m, 5H, Ph); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 19.47, 20.00, 30.61, 32.22, 35.64, 37.13, 41.71, 43.16, 51.50, 125.96, 128.34, 129.29, 141.02, 212.38.

*4.1.2. Ethyl 2-amino-4-benzyl-6-isopropyl-4,5,6,7-tetrahydrobenzo [b]thiophene-3-carboxylate (**9**)*

To a 50 mL round bottom flask, **8** (0.341 g, 1.48 mmols), sulfur (0.048 g, 1.48 mmols), ethylcyanoacetate (0.167 g, 1.48 mmols) and morpholine (0.129 g, 1.48 mmols) were added along with 15 mL of absolute ethanol. The resulting solution was refluxed for 48 h, cooled and then evaporated to dryness *in vacuo*. The resulting crude product was purified by flash silica column chromatography (1:9 ethyl acetate:hexanes) to generate 0.21 g (39%) of **9**. ^{1}H NMR (400 MHz, CD_2Cl_2) δ 0.85 (d, J = 7.2 Hz, 3H, CH_3), 0.89 (d, J = 6.8 Hz, 3H, CH_3), 1.35 (t, J = 5.2 Hz, 3H, CH_3), 1.78–1.89 (m, 2H, CH, CH), 2.45 (t, J = 2.8 Hz, 2H, CH_2), 2.56–2.62 (m, 2H, CH_2, CH_2), 3.15 (dd, J_a = 13.6 Hz, J_b = 2.8 Hz, 1H, CH), 4.41 (q, J = 8.4 Hz, 2H, CH_2), 6.03 (s, 2H, NH_2), 7.20–7.32 (m, 5H, Ph); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 14.93, 19.56, 20.10, 30.64, 32.27, 40.54, 41.75, 43.19, 51.55, 59.74, 118.50, 126.03, 128.41, 129.35, 136.61, 141.05, 141.94, 162.80, 165.63. HRMS (ES+) *m*/*z* (M + H) calcd for $C_{21}H_{27}NO_2S$ 358.1839, found 358.1837 (M + H).

*4.1.3. Ethyl 4-benzyl-6-isopropyl-2-((4-nitrophenoxy) carbonylamino)-4,5,6,7-tetrahydrobenzo[b]-thiophene-3-carboxylate (**10**)*

A solution of dry pyridine (0.16 g, 2.0 mmols) and **9** (0.358 g, 1.0 mmols) in 20 mL of dry CH_2Cl_2 was stirred at room temperature for 30 min. To the reaction, a solution of *p*-nitrophenyl chloroformate (0.303 g, 1.5 mmols), in 5 mL of dry CH_2Cl_2, was added drop wise over a period of 15 min. The reaction was stirred at room temperature for 12 h and then evaporated *in vacuo* to yield the crude product which was purified by flash silica column chromatography (3:7 ethyl acetate:hexanes) to give 0.41 g (77%) of the desired product. ^{1}H NMR (400 MHz, CD_2Cl_2) δ 0.86 (d, J = 6.8 Hz, 3H, CH_3), 0.91 (d, J = 6.8 Hz, 3H, CH_3), 1.46 (t, J = 7.6 Hz, 3H, CH_3), 1.83–1.88 (m, 2H, CH, CH), 2.50–2.82 (m, 4H, CH_2, CH_2), 3.14–3.17 (m, 2H, CH_2), 3.59–3.62 (m, 1H, CH), 4.47 (q, J = 7.2 Hz, 2H, CH_2), 7.16–7.27 (m, 5H), 7.48 (d, J = 8.0 Hz, 2H, Ph), 8.32 (d, J = 8.0 Hz, 2H, Ph), 11.09 (s, 1H, NH); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 14.67, 19.62, 19.99, 28.32, 28.54, 32.36, 35.19, 40.85, 61.25, 111.58, 122.21, 125.37, 126.12, 127.81, 128.35, 129.30, 135.87, 141.30, 145.48, 148.67, 150.12, 155.50, 166.09. HRMS could not be obtained because of decomposition in the mass spectrometer.

*4.1.4. (E)-Ethyl 4-benzyl-2-(3-(3-(4-(tert-butyldimethylsilyl) phenyl)allyl)ureido)-6-isopropyl-4,5,-6,7-tetrahydrobenzo[b] thiophene-3-carboxylate (**11a**)*

Compound **5** (0.250 g, 0.95 mmols) was dissolved in 20 mL of dry THF followed by the addition of pyridine (0.090 g, 1.14 mmols) and DMAP (0.017 g, 0.14 mmols). The resulting solution was stirred for 20 min. A solution of **10** (0.496 g, 0.95 mmols) in 15 mL of dry THF was added drop wise to the reaction mixture and the reaction was stirred for an additional 12–24 h. The reaction was then evaporated to dryness *in vacuo* and the residue was purified by flash silica column chromatography (2:8 ethyl acetate:hexanes) to yield 0.49 g (79%) of the expected products. ^{1}H NMR (400 MHz, CD_2Cl_2) δ 0.86 (d, J = 6.8 Hz, 3H, CH_3), 0.91 (d, J = 6.8 Hz, 3H, CH_3),1.46 (t, J = 7.6 Hz, 3H, CH_3), 1.83–1.88 (m, 2H, CH, CH), 2.50–2.82 (m, 4H, CH_2, CH_2), 3.14–3.17 (m, 2H, CH_2), 3.59–3.62 (m, 1H, CH), 4.47 (q, J = 7.2 Hz, 2H, CH_2), 7.16–7.27 (m, 5H), 7.48 (d, J = 8.0 Hz, 2H, Ph), 8.32 (d, J = 8.0 Hz, 2H, Ph), 11.09 (s, 1H, NH); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 14.67, 19.62, 19.99, 28.32, 28.54, 32.36, 35.19, 40.85, 61.25, 111.58, 122.21, 125.37, 126.12, 127.81, 128.35, 129.30, 135.87, 141.30, 145.48, 148.67, 150.12, 155.50, 166.09. HRMS could not be obtained because of decomposition in the mass spectrometer.

*4.1.5. (E)-Ethyl 4-benzyl-6-isopropyl-2-(3-(3-(4-methoxyphenyl) allyl)ureido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (**11b**)*

Compound **6** (0.097 g, 0.60 mmols) was dissolved in 15 mL of dry THF followed by the addition of pyridine (0.052 g, 0.65 mmols) and DMAP (0.008 g, 0.06 mmols). To the reaction, which was stirred for 20 min, a solution of **10** (0.310 g, 0.60 mmols) in 10 mL of dry THF was added drop wise. The reaction was stirred at room temperature for 12–24 h and evaporated to dryness in *vacuo*. The expected product (0.16 g, 69%) was obtained from the residue after purification by flash silica column chromatography (3:7 ethyl acetate:hexanes). ^{1}H NMR (400 MHz, CD_2Cl_2) δ 0.85 (d, J = 6.8 Hz, 3H, CH_3), 0.90 (d, J = 6.8 Hz, 3H, CH_3), 1.19–1.26 (m, 1H, CH), 1.37 (t, J = 7.6 Hz, 3H, CH_3), 1.41–1.48 (m, 1H, CH), 1.79–1.85 (m, 1H, CH), 2.28 (t, J = 5.6 Hz, 2H, CH_2), 2.55 (d, J = 6.2 Hz, 2H), 3.13 (d, J = 6.0 Hz, 2H, CH_2), 3.76 (s, 3H, CH_3), 4.05 (t, J = 6.0 Hz, 2H, CH_2), 4.41 (q, J = 6.8 Hz, 2H, CH_2), 5.71 (t, J = 4.8 Hz, 1H, NH), 6.08–6.17 (sextet, J = 15.6 Hz, 1H, CH=CH), 6.53 (d, J = 15.6 Hz, 1H, CH=CH), 6.84 (d, J = 8.4 Hz, 2H, Ph), 7.19–7.33 (m, 7H, Ph), 10.80 (s, 1H, NH); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 14.77, 19.66, 19.81, 28.48, 32.83, 35.23, 36.72, 40.87, 42.96, 55.41, 60.68, 108.39, 114.15, 123.76, 125.72, 126.00, 127.75, 128.40, 129.16, 129.44, 131.52, 134.52, 141.62, 152.25, 153.85, 159.60, 166.76. HRMS (ES+) *m*/*z* (M + H) calcd for $C_{32}H_{38}N_2O_4S$ 547.2632, found 547.2631 (M + H)

*4.1.6. (E)-Ethyl 4-benzyl-6-isopropyl-2-(3-(3-(4-methoxyphenyl) allyl)theino[2,3-d]pyrimidine-2,4-dione (**3**)*

Compound **11b** (0.050 g, 0.09 mmols) was refluxed in sodium methoxide $NaOCH_3$ (0.005 g, 0.10 mmols) and 10 mL of methanol for 3 h. The reaction was cooled and neutralized with Dowex 50 H^+ resin which resulted in a white precipitate. The precipitate was dissolved in hot methanol and filtered through a sintered glass funnel. The resulting filtrate was dried and purified using flash silica column chromatography (1:1 ethyl acetate:hexane) to yield 0.035 g (76%) of the final product. ^{1}H NMR (400 MHz, DMSO) δ 0.86 (d, J = 6.8 Hz, 3H, CH_3), 0.91 (d, J = 6.4 Hz, 3H, CH_3), 1.46–1.58 (m, 1H, CH), 1.81–2.12 (m, 1H, CH), 2.26–2.43 (m, 4H, CH_2, CH_2), 2.73 (dd, J_a = 11.6 Hz, J_b = 4.8 Hz, 1H, CH), 3.18 (d, J = 12.0 Hz, 2H, CH_2), 3.73 (s, 3H, CH_3), 4.61 (d, J = 8.0 Hz, 2H, CH_2), 6.15–6.19 (sextet, J = 16 Hz, 1H, CH=CH), 6.48

(d, $J = 16$ Hz, 1H, CH=CH), 6.85 (d, $J = 8.8$ Hz, 2H, Ph), 7.18–7.38 (m, 7H, Ph), 12.18 (s, 1H, NH); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 20.25, 20.41, 27.31, 28.84, 32.61, 35.65, 37.46, 42.09, 55.75, 112.18, 114.66, 122.72, 126.57, 126.82, 128.19, 128.84, 129.65, 129.78, 131.73, 135.63, 141.86, 150.87, 151.07, 158.99, 159.50; HRMS (ES+) m/z (M + H) calcd for $C_{30}H_{32}N_2O_3S$ 501.2212, found 501.2210 (M + H).

*4.1.7. (E)-Ethyl 4-benzyl-2-(3-(3-(4-(hydroxy)phenyl)allyl) theino [2,3-d]pyrimidine-2,4-dione (**1**)*

Compound **11a** (0.100 g, 0.15 mmols) was refluxed in sodium methoxide $NaOCH_3$ (0.010 g, 0.19 mmols) and 15 mL of methanol for 3 h. The reaction was cooled and neutralized with Dowex 50 H^+ resin which resulted in a white precipitate. The precipitate was dissolved in hot methanol and filtered through a sintered glass funnel. The resulting filtrate was dried and purified using flash silica column chromatography (1:1 ethyl acetate:hexane) to yield 0.058 g (77%) of the final product. ^{1}H NMR (400 MHz, DMSO) δ 0.86 (d, $J = 6.8$ Hz, 3H, CH_3), 0.90 (d, $J = 6.8$ Hz, 3H, CH_3), 1.44–1.60 (m, 2H, CH, CH), 2.25–2.42 (m, 4H, CH_2, CH_2), 2.70–2.75 (m, 1H, CH), 3.16 (d, $J = 7.8$ Hz, 2H, CH_2), 4.59 (d, $J = 7.0$ Hz, 2H, CH_2), 6.04–6.14 (sextet, $J = 16$ Hz, 1H, CH=CH), 6.44 (d, $J = 16$ Hz, 1H, CH=CH), 6.67 (d, $J = 8.8$ Hz, 2H, Ph), 7.18–7.38 (m, 7H, Ph), 9.47 (s, 1H, OH), 12.18 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO) δ 20.26, 20.42, 27.31, 28.85, 32.62, 35.66, 37.47, 42.15, 60.44, 112.21, 116.04, 121.49, 126.58, 126.86, 128.04, 128.25, 128.86, 129.79, 132.29, 135.65, 141.86, 150.82, 150.90, 157.78, 158.97; HRMS (ES+) m/z (M + H) calcd for $C_{29}H_{30}N_2O_3S$ 487.2055, found 487.2046 (M + H).

*4.1.8. Ethyl 3-oxo-5-phenylpentanoate (**13**)*

Sodium ethylacetoacetate (**12**) (5 g, 32.87 mmols) was dissolved in 50 mL of dry THF and cooled to 0 °C under argon. Once cooled, a solution (1.6 M in hexane) of n-butyl lithium (21.88 mL, 34.51 mmols) was added drop wise and the reaction was stirred for 30 min at 0 °C to generate the enolate. Benzyl bromide (5.62 g, 32.87 mmols) was then added and the reaction continued stirring for another 90 min at 0 °C. Upon completion, the reaction was poured into a cold solution of saturated potassium hydrogen phosphate and the resulting aqueous solution was extracted with ethyl ether (3 × 100 mL). The combined organic layers were washed with water, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The resulting material was purified by flash silica column chromatography (1:1:8 CH_2Cl_2:ethyl acetate:hexanes) to obtain 4.75 g (66%) of the final product. ^{1}H NMR (400 MHz, CD_2Cl_2) δ 1.25 (t, $J = 7.2$ Hz, 3H, CH_3), 2.87–2.92 (m, 4H, CH_2, CH_2), 3.42 (s, 2H, CH_2) 4.15 (q, $J = 6.8$ Hz, 2H, CH_2), 7.19–7.31 (m, 5H, Ph); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 14.1, 29.52, 44.56, 49.58, 61.46, 126.31, 128.5, 128.64, 141.05, 167.26, 202.11.

*4.1.9. Diethyl 5-amino-3-phenethylthiophene-2,4-dicarboxylate (**14**)*

Compound **13** (3.04 g, 13.8 mmols), sulfur (0.443 g, 13.8 mmols), ethylcyanoacetate (1.56 g, 13.8 mmols) and morpholine (1.2 g, 13.8 mmols) were refluxed in 60 mL of absolute ethanol for 48 h. Upon completion, the solvent was evaporated *in vacuo* and the resulting material was purified by flash silica column chromatography (1:2:7 ethyl acetate:CH_2Cl_2:hexanes) to yield 1.78 g (38%) of the final product. ^{1}H NMR (400 MHz, CD_2Cl_2) δ 1.31 (t, $J = 7.2$ Hz, 3H, CH_3), 1.37 (t, $J = 7.2$ Hz, 3H, CH_3), 2.82 (t, $J = 8.4$ Hz, 2H, CH_2), 3.52 (t, $J = 8.4$ Hz, 2H, CH_2), 4.24 (q, $J = 7.2$ Hz, 2H, CH_2), 4.34 (q, $J = 7.2$ Hz, 2H, CH_2), 6.58 (s, 2H, br, NH_2), 7.19 (q, $J = 4.8$ Hz, 1H, Ph), 7.29 (d, $J = 4.4$ Hz, 4H, Ph); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 14.47, 14.54, 31.25, 36.73, 60.44, 60.73, 107.79, 109.02, 125.96, 128.42, 128.63, 142.64, 151.57, 162.51, 165.91, 166.83. HRMS (ES+) m/z (M + Na) calcd for $C_{18}H_{21}NO_4SNa$ 370.1079, found 370.1083 (M + Na).

*4.1.10. Diethyl 5-((4-nitrophenoxy)carbonylamino)-3-phenethylthiophene-2,4-dicarboxylate (**15**)*

A mixture of dry pyridine (0.12 g, 1.5 mmols) and **14** (0.300 g, 0.86 mmols) in 20 mL of dry CH_2Cl_2 was placed under argon and stirred for 30 min at room temperature. To the solution, *p*-nitrophenyl chloroformate (0.186 g, 0.93 mmols) dissolved in 10 mL of dry CH_2Cl_2 was added drop wise over a period of 15 min and the resulting mixture was stirred at room temperature for 18 h. The solvent was evaporated *in vacuo* and the residue was purified by flash silica column chromatography (2:2:6 ethyl acetate:CH_2Cl_2:hexanes) to give 0.34 g (77%) of the final, pure product. ^{1}H NMR (400 MHz, CD_2Cl_2) δ 1.35 (t, $J = 6.8$ Hz, 3H, CH_3), 1.43 (t, $J = 7.2$ Hz, 3H, CH_3), 2.86 (t, $J = 8.4$ Hz, 2H, CH_2), 3.61 (t, $J = 8.4$ Hz, 2H, CH_2), 4.3 (q, $J = 7.2$ Hz, 2H, CH_2), 4.42 (q, $J = 7.2$ Hz, 2H, CH_2), 7.19–7.34 (m, 5H, Ph), 7.47 (d, $J = 9.2$ Hz, 2H, Ph), 8.31 (d, $J = 9.2$ Hz, 2H, Ph), 11.36 (s, 1H, NH); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 14.33, 30.63, 36.85, 61.2, 61.86, 114.15, 118.61, 122.19, 125.48, 126.1, 128.46, 128.59, 142.17, 145.73, 148.85, 150.24, 153.67, 155.17, 162.25, 166.15. HRMS could not be obtained because of decomposition in the mass spectrometer.

*4.1.11. (E)-Diethyl 3-phenethyl-5-(3-(3-(4-(trimethylsilyloxy) phenyl)allyl)ureido)thiophene-2,4-dicarboxylate (**16a**)*

Compound **5** (0.170 g, 0.65 mmols) was dissolved in 15 mL of dry THF and to the resulting solution, pyridine (0.061 g, 0.78 mmols) and DMAP (0.012 g, 0.10 mmols) were added. The amine solution was stirred at room temperature for 20 min followed by a drop wise addition of a solution of **15** (0.331 g, 0.65 mmols) in 15 mL of dry THF. The reaction was stirred for an additional 12 h and then evaporated *in vacuo*. The residue was purified by flash silica column chromatography (2:8 ethyl acetate:hexanes) to yield 0.33 g (80%) of pure **16a**. ^{1}H NMR (400 MHz, CD_2Cl_2) δ 0.19 (s, 6H, CH_3), 0.98 (s, 9H, *t*Bu) 1.30–1.39 (m, 6H, CH_3), 2.82 (t, $J = 8.0$ Hz, 2H, CH_2), 3.57 (t, $J = 8.0$ Hz, 2H, CH_2), 4.07 (t, $J = 5.8$ Hz, 2H, CH_2), 4.26–4.37 (m, 4H, CH_2, CH_2), 5.49 (t, $J = 5.2$ Hz, 1H, NH), 6.12 (sextet, $J = 15.6$ Hz, 1H, CH=CH), 6.55 (d, $J = 16.4$ Hz, 1H, CH=CH), 6.80 (d, $J = 8.8$ Hz, 2H, Ph), 7.25–7.30 (m, 7H, Ph), 11.04 (s, 1H, NH); ^{13}C NMR (100 MHz, CD_2Cl_2) δ −4.49, 14.44, 25.66, 30.69, 31.91, 32.44, 36.87, 53.67, 60.86, 61.35, 120.15, 120.44, 123.54, 126.02, 127.75, 128.45, 128.59, 129.42,130.56, 131.89, 142.50, 148.59, 153.86, 155.77, 162.88, 166.78. HRMS (ES+) m/z (M + H) calcd for $C_{34}H_{44}N_2O_6SSi$ 637.2755, found 637.2715 (M + H).

*4.1.12. (E)-Diethyl 5-(3-(3-(4-methoxyphenyl)allyl)ureido)-3-phenethylthiophene-2,4-dicarboxylate (**16b**)*

To a solution of **6** (0.173 g, 1.06 mmols), dissolved in 20 mL of dry THF, pyridine (0.092 g, 1.17 mmols) and DMAP (0.013 g, 0.106 mmols) were added and the resulting solution was stirred at room temperature for 20 min. To the amine solution, a solution of **15** (0.543 g, 1.06 mmols) in 15 mL of dry THF was added drop wise and the resulting reaction was allowed to stir for an additional 12 h. Upon completion, the solvent was evaporated *in vacuo* and the resulting mixture was purified by flash silica column chromatography (3:7 ethyl acetate:hexanes) to generate 0.49 g (85%) of **16b**. ^{1}H NMR (400 MHz, CD_2Cl_2) δ 1.32 (t, $J = 7.2$ Hz, 3H, CH_3), 1.37 (t, $J = 7.2$ Hz, 3H, CH_3), 2.83 (t, $J = 8.4$ Hz, 2H, CH_2) 3.60 (t, $J = 8.4$ Hz, 2H, CH_2), 3.8 (s, 3H, CH_3), 4.10 (t, $J = 5.6$ Hz, 2H, CH_2), 4.28 (q, $J = 7.2$ Hz, 2H, CH_2), 4.37 (q, $J = 7.6$ Hz, 2H, CH_2), 5.57 (t, $J = 5.2$ Hz, 1H, NH), 6.07–6.14 (sextet, $J = 15.6$ Hz, 1H, CH=CH), 6.56 (d, $J = 15.6$ Hz, 1H, CH=CH), 6.84 (d, $J = 8.4$ Hz, 2H, Ph), 6.95 (d, $J = 8.8$ Hz, 2H, Ph), 7.17–7.31 (m, 3H, Ph), 8.13 (d, $J = 9.2$, 2H, Ph), 11.14 (s, 1H); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 14.35, 30.58, 36.82, 55.47, 61.26, 61.57, 114.20, 115.94, 126.07, 126.32, 127.79, 128.46, 128.55,129.31, 142.27, 148.87, 154.18, 156.30, 159.70, 162.61, 163.32, 166.82. HRMS (ES+) *m/z* (M + Na) calcd for $C_{29}H_{32}N_2O_6SNa$ 559.1869, found 559.1872 (M + Na).

4.1.13. (E)-Ethyl 3-(3-(4-methoxyphenyl)allyl)-2,4-dioxo-5-phenethyl-1,2,3,4-tetrahydrothieno[2,3-d]pyrimidine-6-carboxylate (**4**)

Compound **16b** (0.385 g, 0.72 mmols) was refluxed in sodium methoxide $NaOCH_3$ (0.043 g, 0.79 mmols) and 30 mL methanol for 3 h. The reaction was cooled and neutralized with Dowex 50 H^+ resin which resulted in a white precipitate. The precipitate was dissolved in hot methanol and filtered through a sintered glass funnel. The resulting filtrate was dried and purified using flash silica column chromatography (3:7 ethyl acetate:hexane) to yield 0.31 g (88%) of the final product. ^1H NMR (400 MHz, DMSO-d_6) δ 1.28 (t, J = 6.8 Hz, 3H, CH_3), 2.78 (t, J = 7.6 Hz, 2H, CH_2) 3.55 (t, J = 8.0 Hz, 2H, CH_2), 3.73 (s, 3H, CH_3), 4.25 (q, J = 7.2 Hz, 2H, CH_2), 4.60 (d, J = 6.0 Hz, 2H, CH_2), 6.11–6.18 (sextet, J = 16 Hz, 1H, CH=CH), 6.52 (d, J = 16 Hz, 1H, CH=CH), 6.87 (d, J = 8.8 Hz, 2H, Ph), 7.19 (t, J = 6.8 Hz, 1H, Ph), 7.25–7.32 (m, 4H, Ph), 7.36 (d, J = 8.8 Hz, 2H, Ph), 12.56 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 14.91, 30.51, 36.47, 42.17, 55.76, 61.33, 114.68, 116.51, 123.02, 126.53, 126.88, 128.17, 128.91,128.97, 129.77, 131.79, 140.27, 142.27, 149.31, 159.48, 160.03, 162.55, 164.69; HRMS (ES+) *m/z* (M + Na) calcd for $C_{27}H_{26}N_2O_5SNa$ 513.1460, found 513.1453 (M + Na).

4.1.14. (E)-Ethyl 3-(3-(4-hydroxyphenyl)allyl)-2,4-dioxo-5-phenethyl-1,2,3,4-tetrahydrothieno[2,3-d]pyrimidine-6-carboxylate (**2**)

Compound **16a** (0.100 g, 0.16 mmols) was refluxed in sodium methoxide $NaOCH_3$ (0.009 g, 0.17 mmols) in 30 mL methanol for 3 h. The reaction was cooled and neutralized with Dowex 50 H^+ resin which resulted in a white precipitate. The precipitate was dissolved in hot methanol and filtered through a sintered glass funnel. The resulting filtrate was dried and purified using flash silica column chromatography (3:7 ethyl acetate:hexane) to yield 0.083 g (90%) of the final product. ^1H NMR (400 MHz, DMSO-d_6) δ 1.28 (t, J = 7.2 Hz, 3H, CH_3), 2.78 (t, J = 8.0 Hz, 2H, CH_2) 3.55 (t, J = 7.8 Hz, 2H, CH_2), 4.26 (q, J = 6.8 Hz, 2H, CH_2), 4.58 (d, J = 6.0 Hz, 2H, CH_2), 6.06 (sextet, J = 15.6 Hz, 1H, CH=CH), 6.47 (d, J = 16 Hz, 1H, CH=CH), 6.69 (d, J = 8.4 Hz, 2H, Ph), 7.17–7.32 (m, 7H, Ph), 9.48 (s, 1H, OH), 12.55 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 14.62, 30.30, 36.33, 42.31, 61.60, 114.01, 115.89, 117.27, 120.99, 126.50, 128.05, 128.20,128.83, 128.90, 132.69, 141.99, 148.87, 150.47, 154.81, 157.67, 159.20, 162.20. HRMS HRMS (ES+) *m/z* (M + Na) calcd for $C_{26}H_{24}N_2NaO_4S$ 499.1304, found 499.1306 (M + Na)

4.1.15. (E)-3-(4-(tert-Butyldimethylsilyloxy)phenyl)acrylic acid (**18**)

p-Hydroxycinnmic acid (**17**) (5.0 g, 30.46 mmols) and imidazole (5.2 g, 76.15 mmols) were dissolved in 25 mL of dry DMF and the solution was cooled to 0 °C. To the chilled solution, *t*-butyldimethylsilylchloride (11.5 g, 76.15 mmols) was added and the reaction was stirred at room temperature for 3 h. The resulting reaction mixture was poured into 125 mL of water. The white, cloudy solution was extracted with ethyl acetate (3 × 100 mL) and the combined organic layers were washed with brine and dried with sodium sulfate. The organic layer was evaporated to dryness and the residue was purified by flash silica column chromatography (3:7 ethyl acetate:hexanes) to yield 8.32 g (98%) of the final product. ^1H NMR (400 MHz, $CDCl_3$) δ 0.23 (s, 6H, CH_3), 0.99 (s, 9H, *t*Bu) 6.33 (d, J = 16 Hz, 1H, CH=CH), 6.85 (d, J = 8.4 Hz, 2H, Ph), 7.46 (d, J = 8.4 Hz, 2H, Ph), 7.74 (d, J = 16 Hz, 1H, CH=CH); ^{13}C NMR ($CDCl_3$) δ −4.16, 18.46, 25.83, 114.77, 120.80, 127.59, 130.24, 146.94, 158.49, 171.23.

4.1.16. (E)-3-(4-(tert-Butyldimethylsilyloxy)phenyl)prop-2-en-1-ol (**19**)

Compound **18** (5.0 g, 17.98 mmols) was dissolved in 25 mL of dry CH_2Cl_2 and the solution was cooled to −78 °C. To the chilled solution, DIBAL (20% solution in toluene, 61 mL, 71.91 mmol) was added drop wise and the reaction was stirred at −78 °C for 8 h. Upon completion, 75 mL of ether was added to the reaction mixture and the mixture was slowly warmed to 0 °C. Once the solution was equilibrated to 0 °C, 2.5 mL of H_2O was added drop wise, followed by 5 mL of 1 N NaOH and an additional 5 mL of H_2O. The solution was then was warmed to room temperature and stirred for an additional 15 min and anhydrous $MgSO_4$ was added slowly with stirring another 15 min until no hydrated $MgSO_4$ aggregates formed. The salts were filtered and filtrate evaporated to obtain the pure product in 2.43 g (59%) yield. ^1H NMR (400 MHz, CD_2Cl_2) δ 0.22 (s, 6H, CH_3), 1.01 (s, 9H, *t*Bu) 2.13 (s, br, 1H, OH), 4.25 (t, J = 4.8 Hz, 2H, CH_2), 6.24 (quintet, J = 16 Hz, 1H, CH=CH), 6.54 (d, J = 16 Hz, 1H, CH=CH), 6.81 (d, J = 8.8 Hz, 2H, Ph), 7.28 (d, J = 8.4 Hz, 2H, Ph); ^{13}C NMR (100 MHz, CD_2Cl_2) δ −4.48, 18.34, 25.67, 63.74, 120.43, 127.14, 127.77, 130.44, 130.51, 155.64.

4.1.17. (E)-(4-(3-Azidoprop-1-enyl)phenoxy)(tert-butyl)dimethylsilane (**20**)

Compound **19** (0.300 g, 1.13 mmols) was dissolved in 10 mL of dry THF followed by the addition of triethylamine (0.172 g, 1.7 mmol). Methanesulfonyl chloride (0.195 g, 1.7 mmols) was added drop wise to the reaction and the reaction was stirred at room temperature for 3 h. The reaction was then quenched by the addition of 20 mL H_2O and the resulting solution was extracted with ethyl acetate (3 × 25 mL). The combined organic layers were then washed with brine and dried over anhydrous Na_2SO_4. The organic layer was evaporated *in vacuo* to obtain the crude product which was used immediately in the next reaction.

The crude mesylated compound was dissolved in dry DMF and sodium azide (0.067 g, 1.64 mmol) was added. The reaction was stirred at room temperature for 5 h and then poured into 50 mL H_2O to generate a white, cloudy solution. The solution was extracted with ethyl acetate (3 × 50 mL) and the combined organic layers were washed with brine and dried over anhydrous Na_2SO_4. The organic layer was evaporated *in vacuo* to dryness and the crude product was purified by flash silica column chromatography (1:9 ethyl acetate:hexanes) to give 0.25 g (82%) of the final product. ^1H NMR (400 MHz, $CDCl_3$) δ 0.22 (s, 6H, CH_3), 1.01 (s, 9H, *t*Bu) 3.93 (d, J = 6.8 Hz, 2H, CH_2), 6.12 (quintet, J = 16 Hz, 1H, CH=CH), 6.60 (d, J = 16 Hz, 1H, CH=CH), 6.83 (d, J = 8.4 Hz, 2H, Ph), 7.30 (d, J = 8.4 Hz, 2H, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ −4.17, 18.47, 53.45, 120.40, 120.52, 128.08, 129.53, 134.58, 156.12.

4.1.18. (E)-3-(4-(tert-Butyldimethylsilyloxy)phenyl)prop-2-en-1-amine (**5**)

Compound **20** (0.200 g, 0.69 mmols) was dissolved in 8 mL of ethanol and Lindlar's catalyst ($Pd/CaCO_3$, 0.043 g, 0.21 mmol) was added to the solution. The reaction was stirred under 1 atm of H_2 for 5 h, filtered through celite and evaporated to dryness in *vacuo*. The resulting product (0.17 g, 93%) was used without any additional purification. ^1H NMR (400 MHz, CD_2Cl_2) δ 0.19 (s, 6H, CH_3), 0.98 (s, 9H, *t*Bu), 1.19 (br, 2H, NH_2), 3.40 (d, J = 5.6 Hz, 2H, CH_2), 6.19 (quintet, J = 15.6 Hz, 1H, CH=CH), 6.42 (d, J = 16 Hz, 1H, CH=CH), 6.78 (d, J = 8.4 Hz, 2H, Ph), 7.25 (d, J = 8.4 Hz, 2H, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ −4.54, 18.29, 25.63, 44.50, 120.35, 127.41, 128.68, 130.12, 130.99, 155.27.

4.1.19. (E)-3-(4-Methoxyphenyl)acrylonitrile (**22**)

A mixture of 4-iodo anisole (**21**) (20 g, 85.46 mmols), acrylonitrile (18.14 g, 342 mmols), $Pd(OAc)_2$ (1.92 g, 8.55 mmols), Bu_4NBr

(5.51 g, 17.1 mmols) and $NaHCO_3$ (14.36 g, 171 mmols) were vigorously stirred in 80 mL of distilled water at 80 °C for 10 h under argon. The reaction mixture was cooled to room temperature and extracted with diethyl ether (3 × 150 mL). The combined organic layer was washed with distilled water, brine and then dried over anhydrous Na_2SO_4. The organic layer was evaporated *in vacuo* and the resulting material purified by silica column chromatography (7:3 CH_2Cl_2:hexane) yield 4.89 g (48%) of the product as a white solid. ^{1}H NMR (400 MHz, CD_2Cl_2) δ 3.83 (s, 3H, CH_3), 5.75 (d, J = 16.8 Hz, 1H, CH=CH), 6.92 (d, J = 8.8 Hz, 2H, Ph), 7.35 (d, J = 16.4 Hz, 1H, CH=CH), 7.42 (d, J = 8.4 Hz, 2H, Ph); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 30.24, 93.91, 114.97, 119.2, 126.93, 129.61, 150.43, 162.64.

4.1.20. (E)-3-(4-Methoxyphenyl)prop-2-en-1-amine (6)

Lithium aluminum hydride (0.751 g, 19.79 mmols) was added to 25 mL of anhydrous ethyl ether and the resulting solution was stirred at room temperature for 20 min. To a solution of the reductant, **22** (3.15 g, 19.79 mmols), dissolved in 15 mL of anhydrous ethyl ether, was added drop wise over a 10 min period. The reaction was stirred at room temperature for an additional 30 min and then was quenched by the addition of (2.5 mL) of15% NaOH. The reaction was extracted with ethyl ether (3 X 100 mL) and the combined organic layers were washed with brine before being dried with anhydrous Na_2SO_4. The organic layer was evaporated *in vacuo* and the resulting crude residue was purified by flash silica column chromatography (1:10:89 NH_4OH: CH_3OH: CH_2Cl_2)to generate the desired pure product (1.95 g, 61%) in good yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.40 (s, 2H, br, NH_2), 3.47 (s, 2H, br, CH_2), 3.81 (s, 3H, CH_3) 6.17–6.22 (quintet, J = 20 Hz, 1H, CH=CH), 6.45 (d, J = 16 Hz, 1H, CH=CH), 6.85 (d, J = 8.4 Hz, 2H, Ph), 7.31 (d, J = 8.4 Hz, 2H, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.93, 44.64, 55.51, 114.2, 127.58, 129.16, 130.2, 159.22.

4.2. MTS assay

The MTS assay was performed using the Cell Titer 96 aqueous cell proliferation assay (Promega). Fibroblast cells derived from a NIH Swiss mouse embryo (NIH-3T3) were obtained from ATCC and maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were grown to 3 passages and approximately 10,000 cells were seeded into each well of a 96-well plate and allowed to incubate overnight to allow cells to attach to the substrate. Individual wells were treated with various concentrations of compounds **1**–**4** (10, 25, 50 and 100 mg/L in DMSO) in 100 μL of DMEM/FBS media and incubated for 24 h. To analyze the viability of the cells, the media was removed and replaced with 100 μL of fresh DMEM media and 20 μL of MTS reagent solution. The resulting assay mixture was incubated for 1 h at 37 °C in a CO_2 incubator and the absorbance of each well was measured at 490 nm. The assay was conducted in four replicants using DMEM/FBS media as negative control and 5% DMSO as positive control. The results are expressed as the mean percentage of cell viability relative to untreated cells. LD_{50} values were determined by Prism software using non-linear regression.

4.3. Hemolysis assay

Sheep whole blood (Lampire Biological Laboratories, 1 mL) was centrifuged at 3000 rpm for 10 min and the separated plasma and white blood cells were discarded. The remaining red blood cells were washed twice with 2–3 mL of TBS buffer (10 mM Tris, 150 mM NaCl, pH 7.0) and diluted to 1% in TBS. The hemolysis assay was performed, in duplicate, in a 96-well microplate plate using DMSO as the negative control and 1% Triton-X as the positive control. In each well, 120 μL of the 1% red blood cell stock was treated with increasing concentrations of **1** or **2**, dissolved in DMSO) and the final volume was adjusted to 150 μL by the addition of TBS. The plate was incubated at 37 °C for 1 h. After incubation, the plate was centrifuged at 3800 rpm for 5 min to collect the red blood cells and the supernantant was analyzed by diluting 20 μL of the supernatent with TBS buffer until the final volume reached 120 μL. The absorbance at 414 nm was measure to determine the release of hemoglobin. The absorbance of negative control solution was set to 0% hemolysis while the absorbance of the 1% Triton-X was set to 100% hemolysis.

4.4. Antibacterial activity

Eight bacterial strains (five Gram-positive and 3 Gram-negative pathogens) were selected from the Anti-Infective Research Laboratory collection. Tested isolates included 3 *S. aureus* (VISA Mu50, MRSA 494, and VRSA MI) strains, *S. pneumoniae* (ATCC 49619), *Enterococcus faecium* (VRE 7303), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *E. aerogenes* (CEF 3978) and *Klebsiella pneumoniae* (CEF 3978). Antibacterial activity of the thieno[2,3-*d*]pyrimidinedione compounds **1**–**4**, **11a**, **16a**, and **16b** was evaluated by determining minimum inhibitory concentration (MIC) values using the broth microdilution method [32] Briefly, strains were grown overnight at 35 °C on Tryptic soy agar plates (TSA, Difco, Detroit, MI). Bacterial inocula (0.5 Mac Farland, i.e. 10^8 CFU/mL) were prepared by suspending few colonies grown onto a TSA plate in sterile normal saline solution. Resulted bacterial suspensions were diluted in Mueller–Hinton broth (MHB, Difco, Detroit, MI) in order to reach an inoculum of 1–2 × 10^6 CFU/mL. A volume of 100 μL of this inoculum (final titer 5 × 10^5 CFU/mL) was added to 100 μL of twofold serial dilutions of compounds **1**–**4** (final concentrations of 0.06–32 mg/L) in Mueller–Hinton broth. Inoculated plates were incubated at 35 °C for 18–24 h, after which the MIC values were visually read as the lowest concentration of compound that prevented bacterial growth.

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