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CHEMICAL SPACE EXPLORATION AROUND THIENO[3,2-d]PYRIMIDIN-4(3H)-ONE SCAFFOLD LED TO A NOVEL CLASS OF HIGHLY ACTIVE CLOSTRIDIUM DIFFICILE INHIBITORS

Xuwei Shao

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by

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Date Submitted: _____ Date Approved: _____

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ABSTRACT

CHEMICAL SPACE EXPLORATION AROUND THIENO[3,2-*d*]PYRIMIDIN-4(3*H*)-ONE SCAFFOLD LED TO A NOVEL CLASS OF HIGHLY ACTIVE *CLOSTRIDIUM DIFFICILE* INHIBITORS

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Clostridium difficile infection (CDI) is the leading cause of healthcare-associated infection in the United States. Therefore, development of novel treatments for CDI is a high priority. Toward this goal, we began in vitro screening of a structurally diverse in-house library of 67 compounds against two pathogenic *C. difficile* strains (ATCC BAA 1870 and ATCC 43255), which yielded a hit compound, 2-methyl-8-nitroquinazolin-4(3*H*)-one (**2**) with moderate potency (MIC = 312/156 μM). Optimization of **2** gave lead compound **6a** (2-methyl-7-nitrothieno[3,2-*d*]pyrimidin-4(3*H*)-one) with improved potency (MIC = 19/38 μM), selectivity over normal gut microflora, CC_{50}s >606 μM against mammalian cell lines, and acceptable stability in simulated gastric and intestinal fluid. Further optimization of **6a** at *C*2-, *N*3-, *C*4- and *C*7-positions resulted in a library of >50 compounds with MICs ranging from 3 – 800 μM against clinical isolates of *C. difficile*. Compound **8f** (MIC = 3/6 μM) was identified as a promising lead for further optimization.

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LIST OF ABBREVIATIONS

CDI	*Clostridium difficile* infection
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
MIC	minimal inhibitory concentration
VAN	vancomycin
FDX	fidaxomicin
MTZ	metronidazole
rt	room temperature
h	hour
Aq	aqueous
MW	microwave
DMF	*N*, *N*-dimethylformamide
DCM	dichloromethane
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
HBTU	2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetra-methyluronium hexafluorophosphate
HSQC	Heteronuclear Single Quantum Coherence spectroscopy

HMBC	Heteronuclear multiple-bond correlation spectroscopy
DMSO-d6	deuterated dimethyl sulfoxide
TMS	tetramethylsilane
HRMS	high resolution mass spectrometry
MBC	minimum bactericidal concentration
BHIS	brain and heart infusion supplemented
CLSI	Clinical & Laboratory Standards Institute
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
HPLC	high pressure liquid chromatography
AUC	area under the curve
NMR	nuclear magnetic resonance
TLC	thin layer chromatography

Chapter I.Introduction

1.1. *Clostridium difficile* infection

Clostridium difficile is a Gram-positive spore-forming anaerobic bacterium that causes diarrhea and serious intestinal conditions. Toxigenic strains of *C. difficile* produce two glycosylating toxins: toxin A (TcdA/enterotoxin) and toxin B (TcdB/cytotoxin), both of which initiate damage of the colon, life-threatening inflammation of the gut (*C. difficile* colitis), and a spectrum of intestinal pathologies ranging from mild diarrhea to pseudomembranous colitis in the infected host.[1-3] The CDI, confined to the gastrointestinal tract, is usually triggered by the use of antibiotics, which disturbs the reproduction of normal and protective gut microflora allowing *C. difficile* to proliferate in the colon and to produce toxins.[4] According to Centers for Disease Control and Prevention (CDC), about half a million cases of CDI occur each year in the US hospitals and long-term health care facilities with an estimation of 29,000 deaths. The prevalence and severity of CDI appear to be rising, partly due to a larger elder population with high risk factors, an increasing use of antibiotics, a higher proportion of hypervirulent bacterial isolates with increased production of lethal toxins A and B,[5] and the emergence of hypervirulent epidemic strains (BI/Nap1/027).[6]

1.2. Therapeutic options available for CDI

However, therapeutic options available for CDI patients are limited. In 2010, the Infectious Diseases Society of America provided clinical practice guidelines for CDI, in which metronidazole, vancomycin and fidaxomicin are recommended.[7] Metronidazole diffuses into the organism, inhibits protein synthesis by interacting with DNA and causing a loss of helical DNA structure and strand breakage. Therefore, it causes cell death in susceptible organisms.

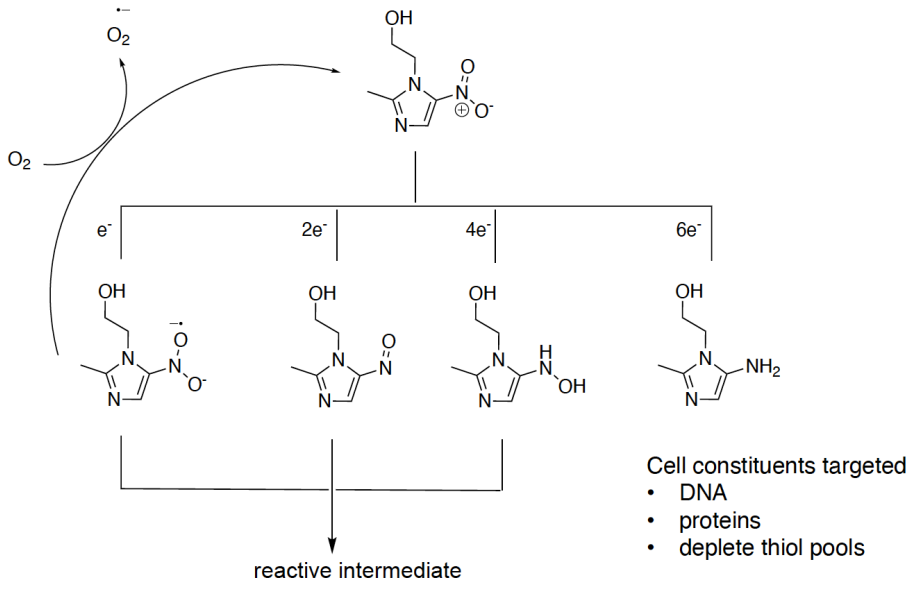

Figure 1. Products of metronidazole reduction. Modified based on previous report.[8,9]

Multiple steps are required for metronidazole molecules to perform their therapeutic function. After diffusing across the cell membranes of anaerobic and aerobic pathogens, nitro group on the molecules is reduced to form free radicals with cytotoxic effect (**Figure 1**). Hypoxic condition is required during the reduction, which explains the selectivity of metronidazole towards anaerobic *C. difficile* strains.[10] The formed free radicals interact with various constituents in the cells, and lead to cell death.[8]

Since approved by the US FDA, vancomycin has been an effective therapeutic option for CDI patients.[11] In Gram-positive bacteria, vancomycin inhibits formation of polymer of glycopeptide (**Figure 2**). Because of the absence of peptide crosslink, peptidoglycan layer becomes less rigid and more permeable, and eventually the bacterium bursts from osmotic lysis.[12]

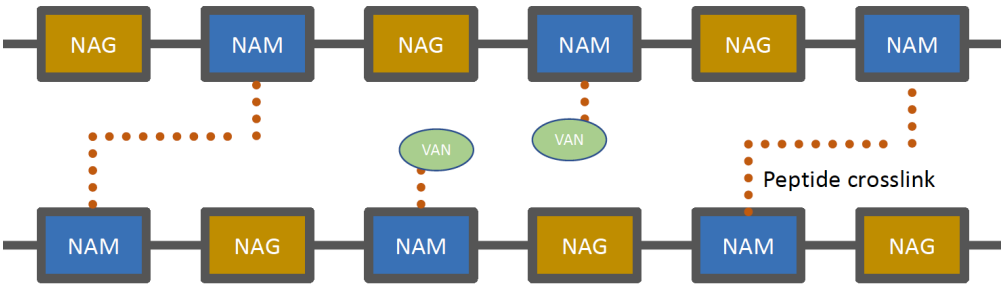

Figure 2. Mechanism of action of vancomycin. Modified based on previous report.[13]

On the contrary to metronidazole or vancomycin, inhibitory activity of fidaxomicin against *C. difficile* strains is the result of blockage of RNA polymerase activity.[14] To initialize transcription process, specific promoter σ combines with the core RNA polymerase (RNAP), which contains α-dimer, β-, β′-, and ω-subunits. Then the complex recognizes and binds to DNA template, which leads to the formation of open RNAP-DNA complex. One of the proposed mechanisms of action of fidaxomicin is that it binds to β′-subunit and σ factor, which blocks formation of the essential component (**Figure 3**).[15]

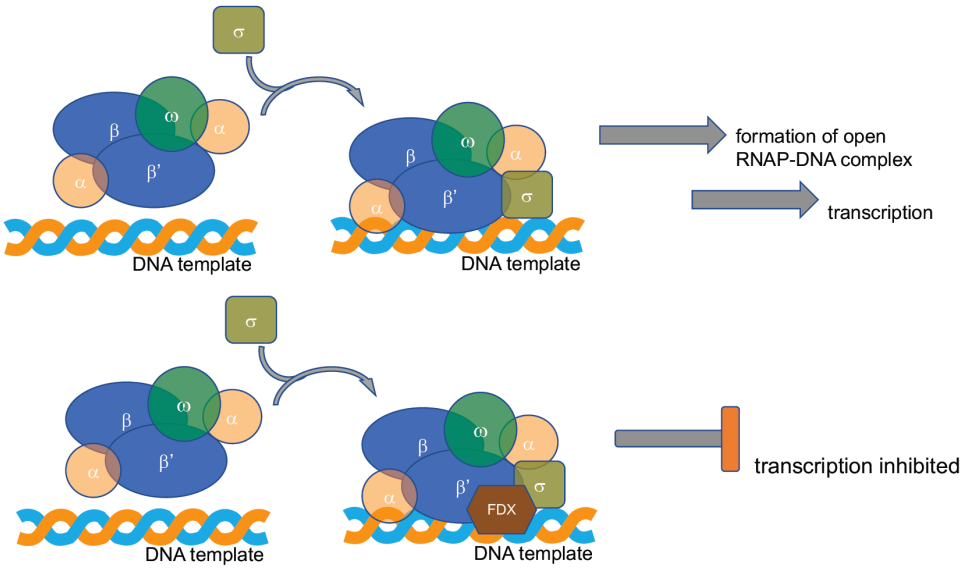

Figure 3. Mechanism of action of fidaxomicin. Modified from previous report.[13,15]

1.3. Drawbacks of medications used for CDI

Currently available therapeutics (metronidazole, vancomycin and fidaxomicin) for CDI are inadequate in efficacy and/or tolerability.[3] Both metronidazole and vancomycin treatments encounter substantial disease relapse.[16] Metronidazole, an antibiotic with activity against a wide spectrum of anaerobic bacteria and parasites, is only recommended for the treatment of mild-to-moderate episodes and is inferior to vancomycin.[17] Moreover, it is essentially 100% bioavailable leading to limiting concentrations in the colon, the prime location of CDI.[18] Unlike metronidazole, vancomycin is minimally absorbed into the systemic circulation upon oral administration, thereby resulting in a high concentration in the colon.[19] However, its broad spectrum of action against Gram-positive bacteria leads to a reduced microbiome diversity and the potential selection of vancomycin-resistant enterococci.[20] In addition, recurrent infection caused by newer and stronger *C. difficile* strains is a formidable preclinical challenge.[21] Both metronidazole and vancomycin treatments can worsen the condition of patients due to the loss of beneficial gut microbiota, and subsequent recurrence at an alarming rate. Selectively targeting *C. difficile* over normal gut flora has been considered as a strategy to achieve prevention of recurrence.[22] Compared with vancomycin, fidaxomicin (a macrolide antibiotic) demonstrates a narrower spectrum of activity and selectivity towards *C. difficile*; however, it does not greatly improve sustained clinical responses especially against hypervirulent strains BI/NAP1/027.[23] In view of the transient efficacy of these antibiotics, particularly of metronidazole and vancomycin, patients are predisposed to ~25% relapse rate as compared to 15% for fidaxomicin and a subsequent prolongation of *C. difficile* shedding and transmission.[24, 25] Although fidaxomicin treatment showed significantly lower rates of CDI recurrence compared to metronidazole and vancomycin, it does so only in non-NAP1 CDI patients.

In addition, clinical resistance to fidaxomicin has already been documented.[24] Although 93% of fidaxomicin remains unabsorbed after oral administration, it is detectable in the range of 25-50 ng/mL in the plasma of patients,[26] which leads to a serious concern of potent cytotoxic effect. Moreover, the cost of fidaxomicin treatment is prohibitively expensive partly due to complexity in synthesizing a large molecule with molecular weight beyond 1000 Da. Although ridinilazole (NCT02784002) is currently undergoing clinical trials for the treatment of CDI, it remains to be seen whether it would offer any benefit over current treatment.[27] Despite unmet medical need, progress toward anti-*C. difficile* drug development has been very limited.[28-32] Therefore, the discovery of new "best-in-class" drugs to fight against *C. difficile* is needed to adequately address CDI.

Chapter II. Design rationale

To identify highly selective novel *C. difficile* inhibitors, we conducted whole-cell screening of a set of 67 in-house compounds (**Table 1**), comprising diverse structural classes (valine-, proline-, phenylalanine-, and tyrosine-derived thiazole peptidomimetics and quinazolinones, benzoxazines, indazoles, benzodioxines, imidazopyridines, and benzodioxepines) with molecular weights (MW) ranging from 164 to 652 Da.

Table 1. List of in-house compound library used for HTS

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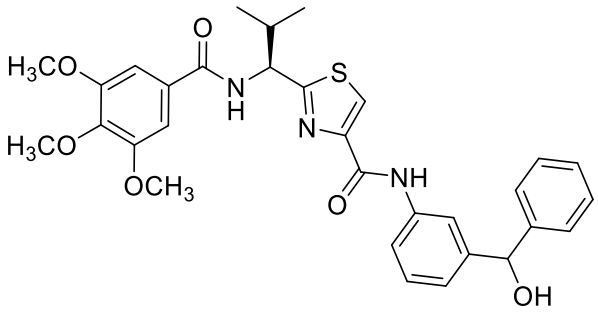
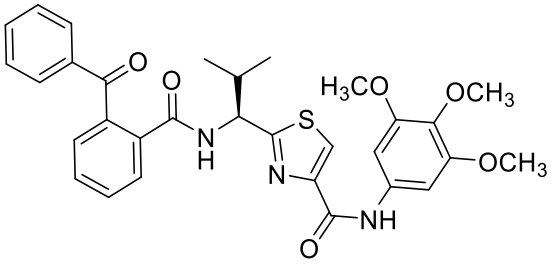
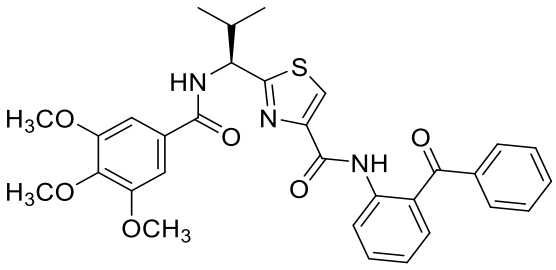
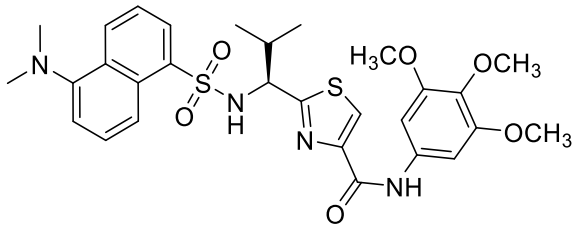
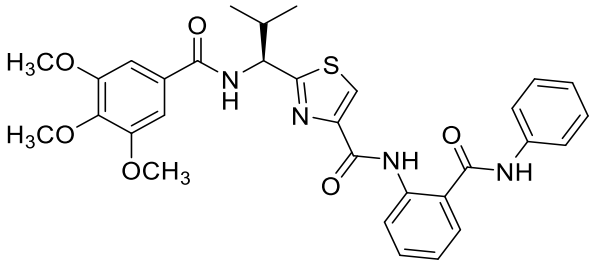
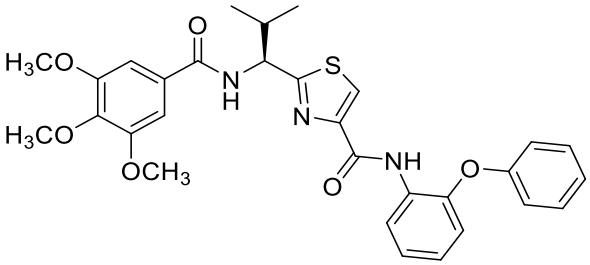
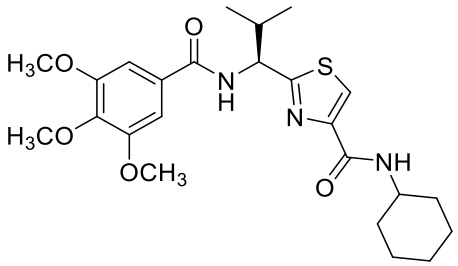
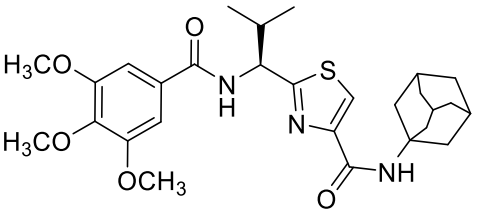
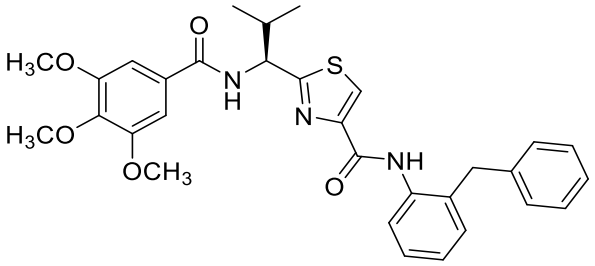
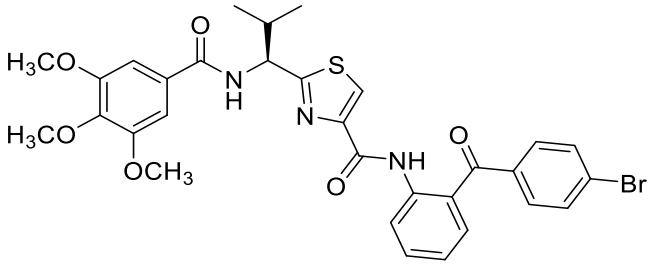
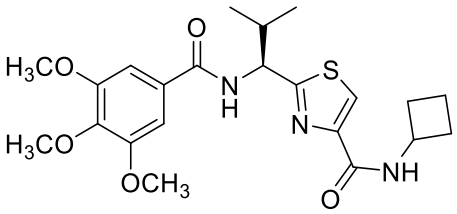
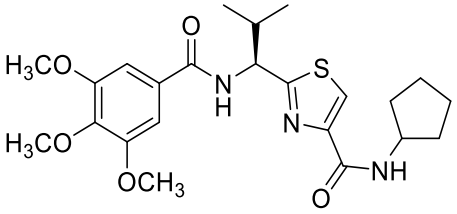
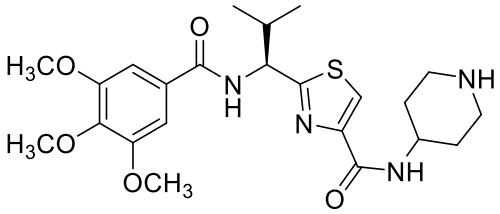
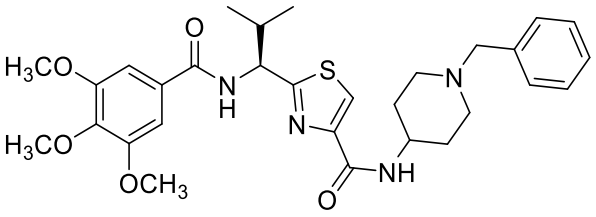
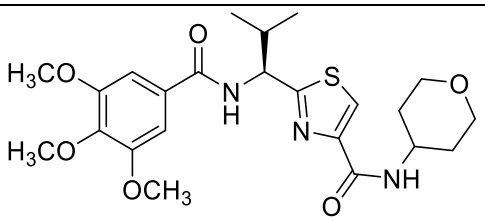
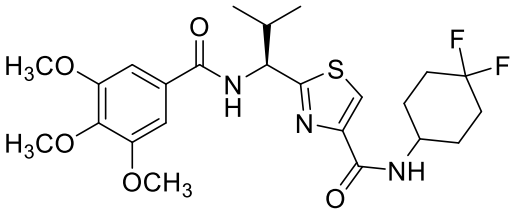
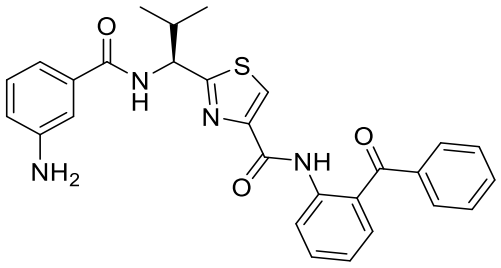
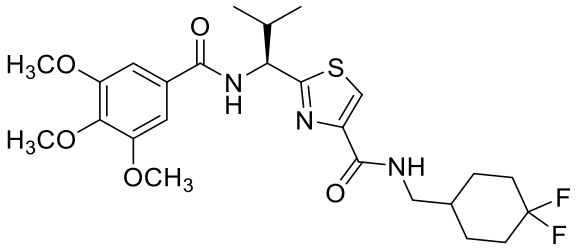
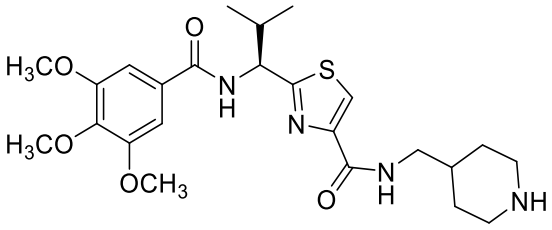
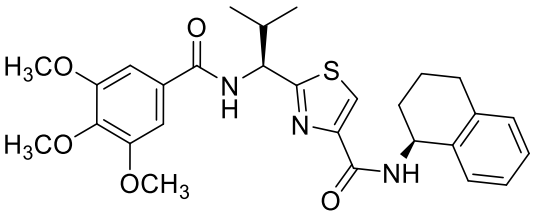
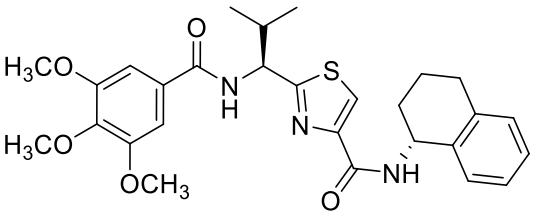
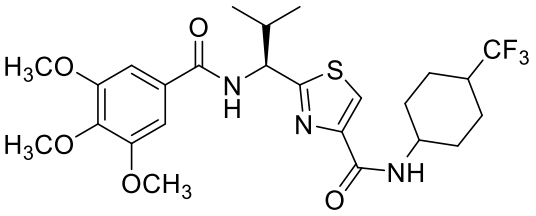
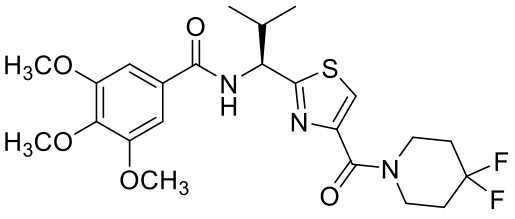
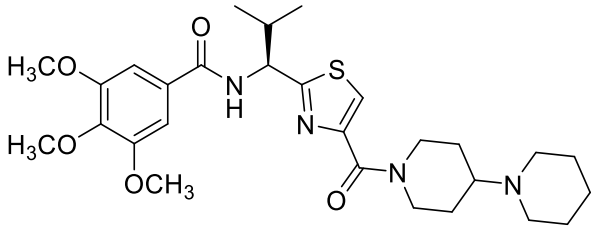
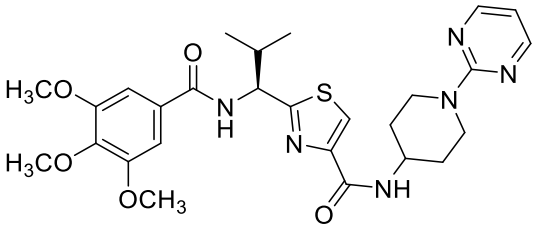
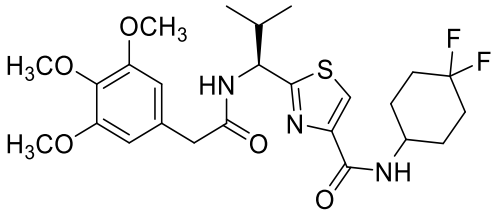
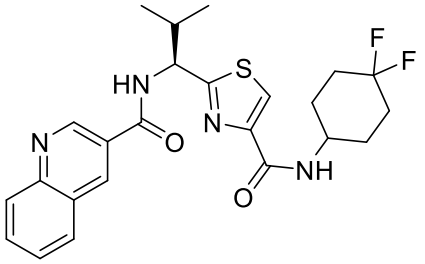
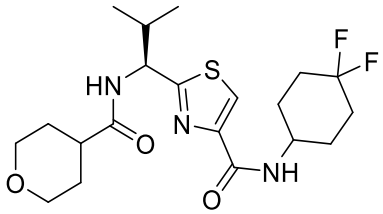
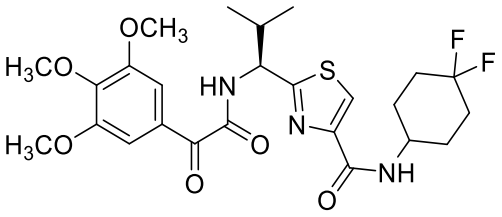
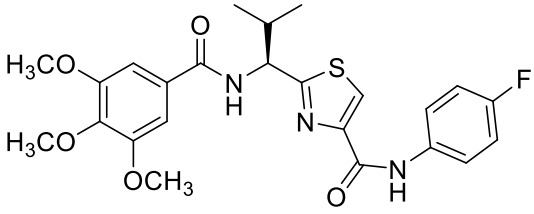
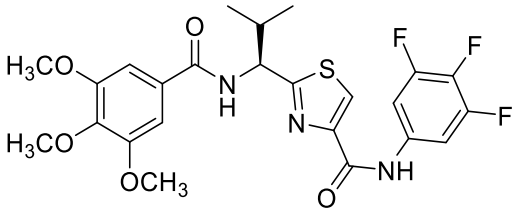
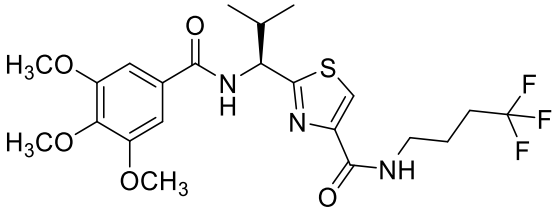
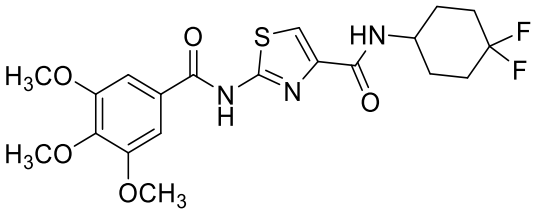
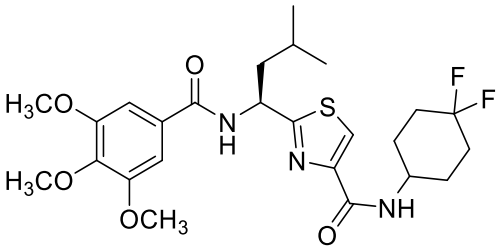
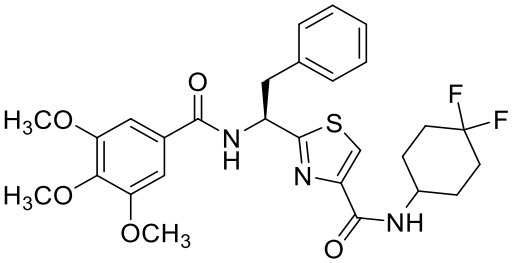
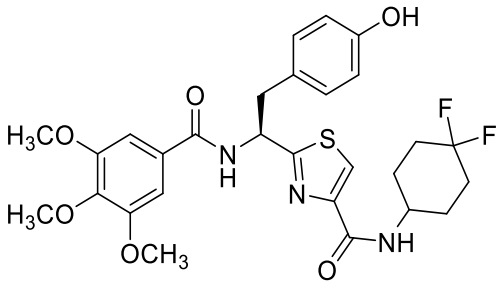
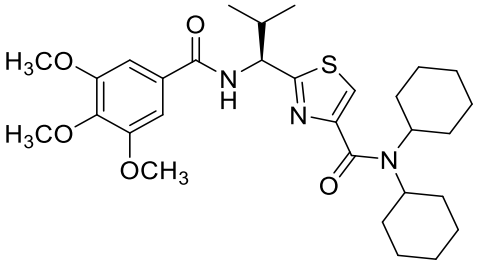
HTS-1	
HTS-2	
HTS-3	

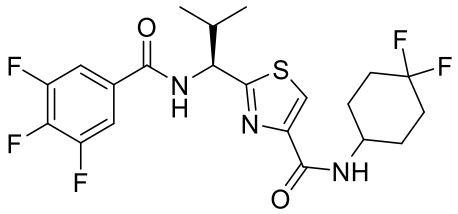
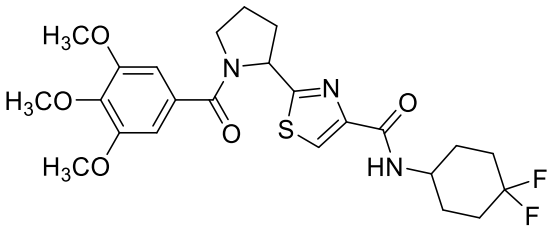
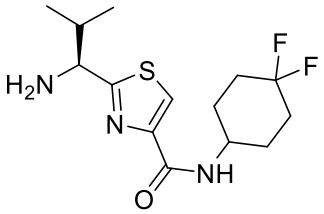
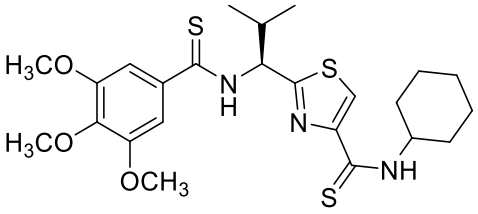
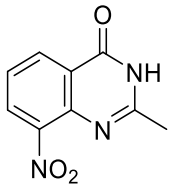
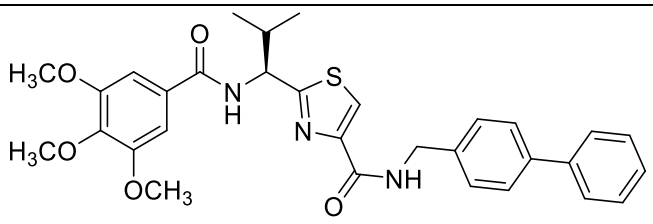
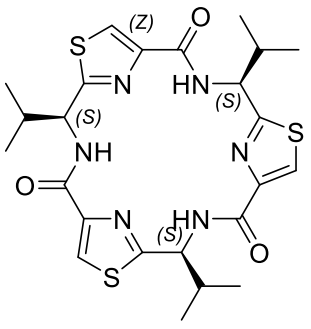
HTS-4	
HTS-5	
HTS-6	
HTS-7	
HTS-8	
HTS-9	

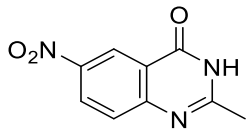
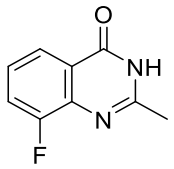
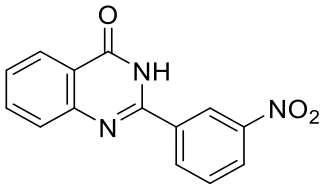
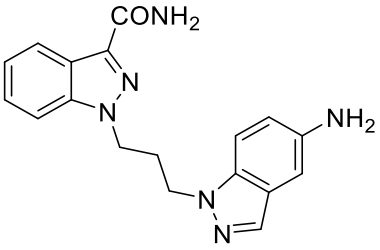
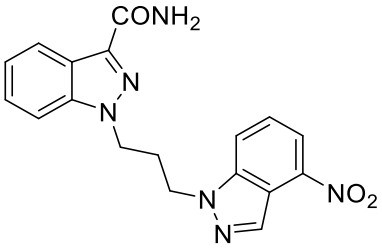
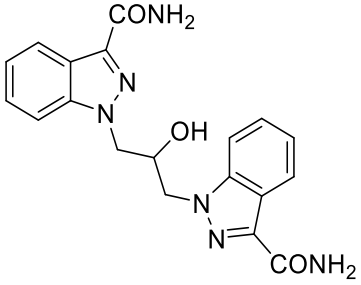
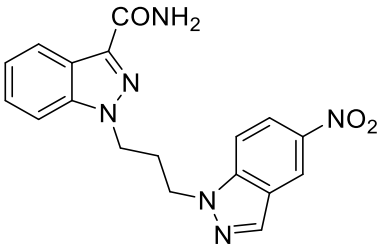
HTS-10	
HTS-11	
HTS-12	
HTS-13	
HTS-14	
HTS-15	
HTS-16	

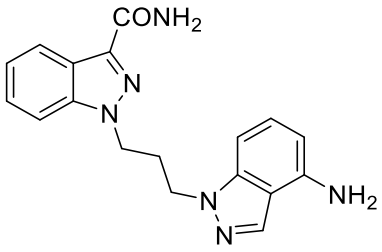
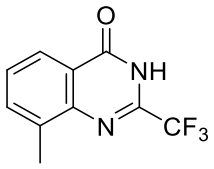
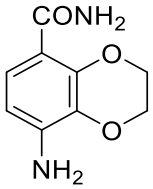
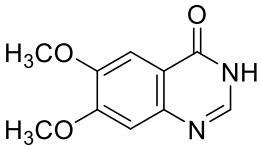
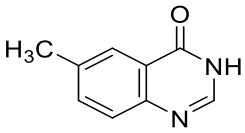
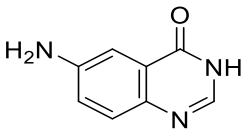
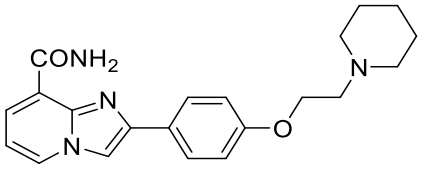
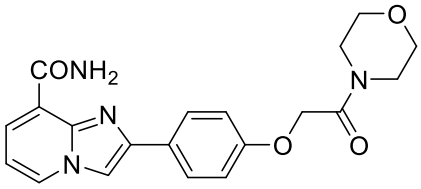
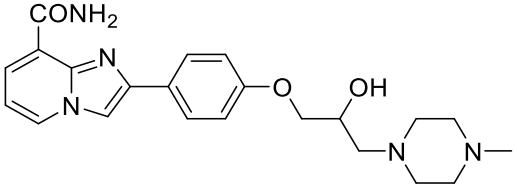
HTS-17	
HTS-18	
HTS-19	
HTS-20	
HTS-21	
HTS-22	
HTS-23	

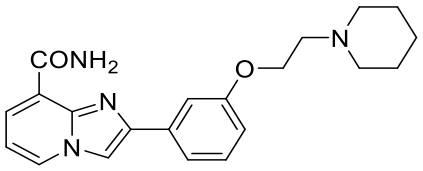
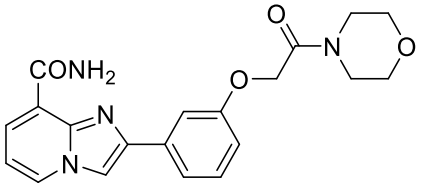
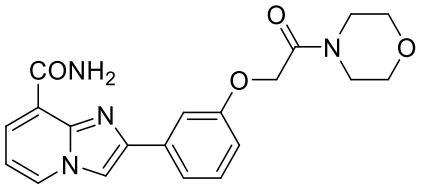
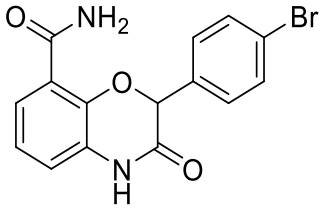
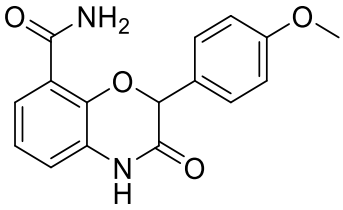
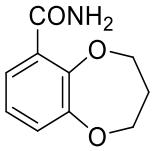
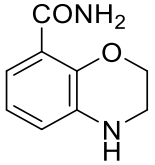
HTS-24	
HTS-25	
HTS-26	
HTS-27	
HTS-28	
HTS-29	
HTS-30	

HTS-31	
HTS-32	
HTS-33	
HTS-34	
HTS-35	
HTS-36	
HTS-37	

HTS-38	
HTS-39	
HTS-40	
HTS-41	
HTS-42	
HTS-43	
HTS-44	

HTS-45	
HTS-46	
HTS-47	
HTS-48	
HTS-49	
HTS-50	
HTS-51	

HTS-52	
HTS-53	
HTS-54	
HTS-55	
HTS-56	
HTS-57	
HTS-58	
HTS-59	
HTS-60	

HTS-61	
HTS-62	
HTS-63	
HTS-64	
HTS-65	
HTS-66	
HTS-67	

This screening method allowed us to ensure penetration of the *C. difficile* cell membrane as well as to obtain minimum inhibitory concentrations (MICs) as biological readouts to identify promising hit compounds. Each scaffold in the library had a sufficient number of structurally close analogues to produce robust results while capturing key SAR trends. This screening test identified two previously reported quinazolinone analogues[33,34] as hit compounds, 6-nitroquinazolin-4(3*H*)-one (**1**, MIC =

335/335 μM, hereafter, against two *C. difficile* clinical strains; ATCC BAA 1870/ATCC 43255) and 2-methyl-8-nitroquinazolin-4(3*H*)-one (**2**, MIC = 312/156 μM) (3% success rate) that showed moderate to weak MICs. In vitro MIC values were compared with three FDA approved drugs, vancomycin, metronidazole and fidaxomicin. Compound **2** was established as a promising fragment hit for further medicinal chemistry optimization because of its selectivity profile toward multiple clinical strains of *C. difficile* over human normal microflora (MIC >1248 μM) such as *Lactobacillus*, *Bifidobacterium*, *Escherichia coli*, and *Enterobacter cloacae*) (**Figure 4**). Medicinal chemistry optimization of hit **2** via analogue synthesis produced compounds **3**-**5** with a loss of potency suggesting the contribution of the *C*8-nitro substituent. Next, we decided to implement scaffold hopping strategy, which led to identification of small MW scaffolds **6a** (MIC = 19/38 μM), **6b** (MIC = 41/41 μM) and **6c** (MIC = 38/38 μM). Compound **6a** was prioritized for further SAR study based on its structural novelty, ease of synthetic derivatization, favorable potency, selectivity, and in vitro mammalian cell toxicity as shown in Figure 4.

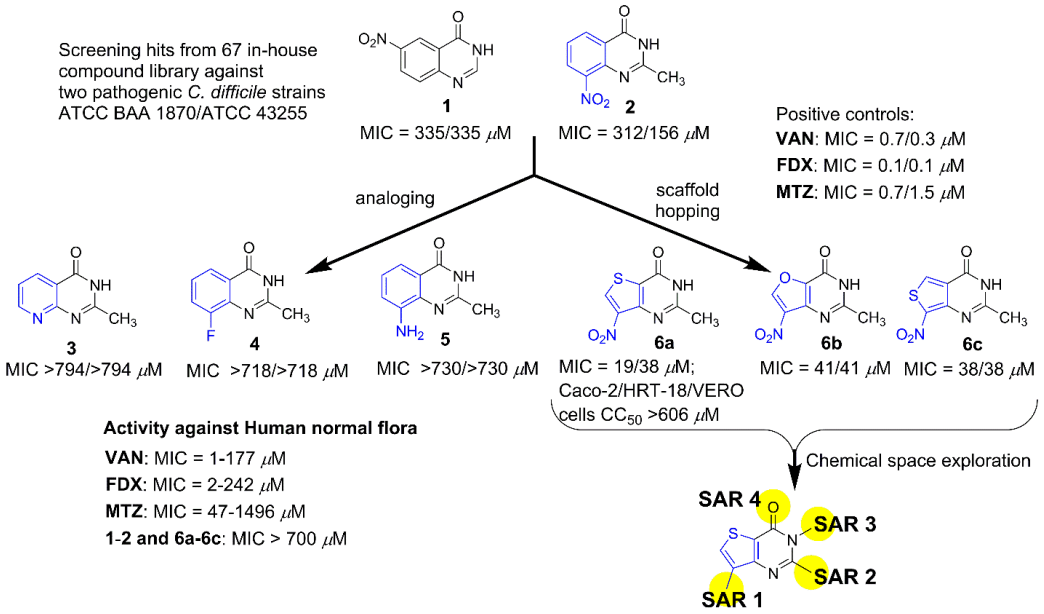


Figure 4. Hit identification.

Chapter III. Experimental

3.1. Chemicals and Bacterial Strains

Chemical reagents and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI), AK scientific (Union City, CA), Combi-Blocks Inc. (San Diego, CA), TCI America (Portland, OR), Gold Biotechnology (St. Louis, MO), Alfa Aesar (Ward Hill, MA) and Sigma-Aldrich (St. Louis, MO), and were used as received.

Table 2. Bacterial strains used in the study

Bacterial strain	ID number	Comments
Clostridium difficile 4118	ATCC BAA-1870	Clinical isolate from Main, USA. Toxinotype IIIb, ribotype 027, *tcdA*+ and *tcdB*+
Clostridium difficile VPI 10463	ATCC 43255	Clinical isolate from abdominal wound. Toxinotype 0, ribotype 087, *tcdA*+ and *tcdB*+.
Lactobacillus crispatus JV-V01	HM-103	Isolated from normal human vaginal flora.
Lactobacillus casei Hansen and Lessel	ATCC 334	Isolated from Dairy products; emmental cheese.
Lactobacillus gasseri EX336960VC03	HM-400	Isolated from human mid-vaginal wall in March 2010 in Richmond, Virginia.
Bifidobacterium bifidum 212A	ATCC 11863	---
Escherichia coli Castellani and Chalmers	ATCC 25922	Standard FDA strain for antibiotic susceptibility testing
Enterobacter cloacae	ATCC BAA-1143	Quality control strain

Bacterial strains (**Table 2**) were purchased from American Type Culture Collection (ATCC) or Biodefense and Emerging Infections Research Resources Repository (BEI resources). Bacterial media were purchased from Becton, Dickinson and Company (Cockeysville, MD) while cell culture media and fetal bovine serum were purchased from Fisher scientific (Waltham, MA).

3.2. Chemistry-General

All chemicals reagents were confirmed for uniformity by thin layer chromatography (TLC) with silica gel as the adsorbent layer (250 microns) on aluminum backed plates (Agela Technologies, Torrance, CA). Reactions were monitored by TLC and visualized by ultraviolet (UV) light at 254 nm. ^{1}H NMR spectra (at 400 MHz) and ^{13}C NMR spectra (at 100 MHz) were recorded on a Bruker 400 UltrashieldTM spectrometer. Chemical shifts (δ) of ^{1}H NMR and ^{13}C NMR were reported downfield from tetramethylsilane (TMS, internal standard) in parts per million (ppm) units. The ^{1}H NMR data are presented as follows: chemical shift [multiplicity s (singlet), bs (broad singlet), d (doublet), t (triplet), q (quartet), hept (heptet), dd (doublet of doublets), and m (multiplet), number of protons, coupling constant]. The ^{13}C NMR (proton decoupled, fluorine coupled) data are presented as follows: chemical shift [multiplicity d (doublet)]. Flash chromatography was carried out on a Reveleris X2 flash chromatography system (Buchi Corporation, New Castle, DE). Preparative TLC was used for the purification of certain target compounds using Silica Gel GF 1000 μm 20x20 cm glass backed plates from Analtech (Miles Scientific, Newark, DE). Purity of the target compounds was established by HPLC analysis using Waters 600 HPLC system with a Waters 717 plus autosampler, Waters 2487 dual 18λ absorbance detector at 254 nm (Waters, Milford, MA) and a C18 reverse phase column (Luna® 5 μm C18 100 Å, LC Column 150 x 4.6 mm) at respective flow rate. Purity of the target compounds was determined to be ≥ 95%

based on HPLC analysis. The purity of all target compounds was determined by the ratio of major peak area to the total combined area of peaks. Melting points were determined using Stuart digital melting point apparatus SMP20 (Cole-Parmer, Staffordshire, UK) and are uncorrected. Mass spectra were recorded for known target compounds using an Agilent 1260 infinity series liquid chromatography (LC) system (C18 column, Agilent InfinityLab poroshell 120, EC-C18, 2.7 μm 4.6 x 50 mm) connected with Agilent 6120 quadrupole mass spectrometer (MS). Aqueous solubility in PBS buffer and stability in simulated gastric fluid and simulated intestinal fluid were determined using Waters 600 HPLC system with a Waters 717 plus autosampler, Waters 2487 dual 19λ absorbance detector (Waters, Milford, MA) and a C18 reverse phase column (Symmetry C18, 5 μm, 3.9 x 150 mm) at a flow rate of 1 mL/min. The X-ray intensity data were measured on a Bruker APEX-II CCD system equipped with a graphite monochromator and a Mo sealed tube (λ = 0.71073 Å). High resolution mass spectra (HRMS) were obtained for all unknown target compounds from the Columbia University Chemistry Department Mass Spectrometry Facility on a Waters Xevo G2-XS QToF mass spectrometer equipped with H-Class UPLC inlet and a LockSpray ESI source.

3.3. Synthetic procedure

3.3.1. General Procedure for Synthesis of Thienopyrimidinone Analogues (Method A).[34]

Commercially available amino and methyl ester substituted aromatic/heteroaromatic compound (1 eq mmol) was added to a flask containing acetic anhydride (20 mL). After stirring at room temperature for 12 to 24 h, excess liquid was removed under vacuum. The crude product was then purified by flash chromatography with dry loading method as described below. The crude residue was dissolved in a mixture of acetone and

methanol (50:50) and 8 g of silica gel was added into the same flask. Completely dried solid was transferred into a solid loader and installed onto flash chromatography. Without further purification unless otherwise indicated, respective intermediates were stirred in 30% aqueous solution of ammonium hydroxide (30 mL) at rt until a complete conversion of starting material was observed by TLC. During the process, the mixture turned into a clear solution from a suspension. Ammonia was first released at a lower temperature under vacuum, and then water was evaporated at a higher temperature. A solid product was obtained by flash chromatography using dry loading method and dichloromethane/methanol as an eluent system.

3.3.2. General Procedure for the Insertion of a Nitro Group on the Core Structures (Method B).[35]

Concentrated sulfuric acid (10 mL) was cooled down to 0 °C on ice bath. Respective scaffold (1 eq) was added and stirred for half an hour before drop wise addition of fuming nitric acid (1 mL). The resulting mixture was stirred for half an hour at 0 °C and 8 h at rt to produce yellow solution, which was then poured into excess ice water and neutralized with $NaHCO_3$ to pH 7. The solid precipitates were filtered and washed with water.[36]

3.3.3. General Procedure for the Synthesis of C2-styryl Derivatives of Thienopyrimidinone Core (Method C).[37,38]

To a 0.5-2 mL microwave vial, compound 6a (1 eq), (un)substituted aromatic/heteroaromatic aldehydes (5 eq), and acetic acid (2 mL) were added. After the vial was crimped, the mixture was subjected to irradiation and the temperature was maintained at 180 °C for 1 h in a mono-cavity microwave initiator. After heating, compressed air was used to cool down the reaction mixture. The process was repeated

5 times and the resulting mixture was purified by flash chromatography using dry loading method and dichloromethane/methanol as an eluent system.

3.3.4. General Procedure for Alkylation at *N*3-position of Thienopyrimidinone Core (Method D).[39]

Compound **6a** (1 eq) was reacted with (un)substituted phenylalkyl halide or halo acetate (1.8 eq) in the presence of K_2CO_3 (2 eq) in *N*,*N*-dimethylformamide (15 mL) at rt for 8-12 h. After complete consumption of starting material monitored by TLC, the mixture was diluted with ethyl acetate (100 mL) and washed with saturated aqueous solution of $NaHCO_3$ and brine three times each. Organic layer was dried over anhydrous $MgSO_4$ and evaporated under vacuum. The crude product was purified by flash chromatography using dry loading method and dichloromethane/methanol as an eluent system.

3.3.5. 2-Methyl-8-nitroquinazolin-4(3*H*)-one (2).[34] Intermediate 2-methyl-8-nitro-4*H*-benzo[*d*][1,3]oxazin-4-one **17** (206 mg, 1.0 mmol) was used as starting material to prepare compound **2** according to method A as an orange solid (123 mg, 60% yield), mp 247-249 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 12.62 (s, 1H), 8.27 (dd, J = 24.6 Hz, 7.4 Hz, 2H), 7.59 (t, J = 7.7 Hz, 1H), 2.37 (s, 3H). ESI-MS: m/z 206.1 $[M + H]^+$. HPLC flow rate 0.5 mL/min, t_R (acetonitrile/water 90:10) = 5.9 min, purity 99%.

3.3.6. 2-Methylpyrido[2,3-*d*]pyrimidin-4(3*H*)-one (3).[40] Intermediate 2-methyl-4*H*-pyrido[2,3-*d*][1,3]oxazin-4-one **18** (162 mg, 1.0 mmol) was used as the starting material to synthesize target compound **3** according to method A as a white solid (78 mg, 48% yield), mp 275-278 °C [lit. mp 261-263 °C].[41] ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 12.50 (s, 1H), 8.90 (q, J = 5.2 Hz, 1H), 8.46 (dd, J = 12.0 Hz, 3.2 Hz, 1H), 7.48 (q, J = 17.2 Hz, 1H), 2.40 (s, 3H). ESI-MS: m/z 162.1 $[M + H]^+$. HPLC flow rate 0.5 mL/min, t_R (acetonitrile/water 90:10) = 4.2 min, purity 99%.

3.3.7. 8-Fluoro-2-methylquinazolin-4(3*H*)-one (4).[34, 42] Intermediate 8-fluoro-2-methyl-4*H*-benzo[*d*][1,3]oxazin-4-one **19** (179 mg, 1.0 mmol) was used for the preparation of **4** according to method A as a white solid (121 mg, 68% yield), mp 273-275 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 12.45 (s, 1H), 7.94 (d, J = 7.9 Hz, 1H), 7.73 – 7.68 (m, 1H,), 7.54 – 7.49 (m, 1H), 2.36 (s, 3H). ESI-MS: m/z 179.1 $[M + H]^+$. HPLC flow rate 1 mL/min, t_R (acetonitrile/water 65:35) = 5.7 min, purity 97%.

3.3.8. 8-Amino-2-methylquinazolin-4(3*H*)-one (5).[42] To a 50 mL beaker, compound **2** (205 mg, 1.0 mmol), Pd/C (20 mg) and ethanol (20 mL) were added and the beaker was transferred into a Parr-hydrogenation vessel. Upon three times replacements of air by nitrogen, hydrogen gas was introduced until 50 psi. After no further consumption of hydrogen was observed, reaction mixture was removed and filtered through celite. Compound **5** was collected upon concentration under vacuum as a yellow solid (123 mg, 70% yield), mp 232-234 °C [lit. mp 226-230 °C].[34] ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 12.05 (s, 1H), 7.19 (dd, J = 7.7 Hz, 1.4 Hz, 1H), 7.11 (t, J = 15.3 Hz, 1H), 6.93 (dd, J = 7.7 Hz, 1.2 Hz, 1H), 5.57 (s, 2H), 2.34 (s, 3H). ESI-MS: m/z 176.1 $[M + H]^+$. HPLC flow rate 1 mL/min, t_R (acetonitrile/water 65:35) = 4.9 min, purity 99%.

3.3.9. 2-Methyl-7-nitrothieno[3,2-*d*]pyrimidin-4(3*H*)-one (6a). Nitro group was introduced onto **7a** (166 mg, 1.0 mmol) according to method B to yield **6a** as a white solid (158 mg, 75% yield), mp 249-252 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 12.90 (s, 1H), 9.31 (s, 1H), 2.44 (s, 3H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 159.23, 157.84, 149.26, 141.31, 138.99, 122.05, 21.87. HRMS (m/z): $[M + H]^+$ calcd for $C_7H_6N_3O_3S$, 212.0124; found: 212.0099.

3.3.10. 2-Methyl-7-nitrofuro[3,2-*d*]pyrimidin-4(3*H*)-one (6b). Compound **7b** (150 mg, 1.0 mmol) was subjected to nitration according to method B to obtain **6b** as a white solid (160 mg, 82% yield), mp 270-273 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ

12.98 (s, 1H), 8.05 (s, 1H), 2.39 (s, 3H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 158.39, 154.56, 152.56, 147.18, 137.32, 108.19, 21.73. HRMS (*m/z*): $[M + H]^+$ calcd for $C_7H_6N_3O_4$, 196.0353; found: 196.0333.

3.3.11. 2-Methyl-7-nitrothieno[3,4-*d*]pyrimidin-4(3*H*)-one (6c). Nitration of **7c** (166 mg, 1.0 mmol) using method B gave **6c** as a white solid (84 mg, 40% yield), mp 256-258 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 12.43 (s, 1H), 8.84 (s, 1H), 2.39 (s, 3H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 161.18, 157.40, 147.01, 138.43, 135.17, 125.67, 22.41. HRMS (*m/z*): $[M + H]^+$ calcd for $C_7H_6N_3O_3S$, 212.0124; found: 212.0131.

3.3.12. 7-Nitrothieno[3,2-d]pyrimidin-4(3H)-one (6d).[43] Compound **7d** (152 mg, 1.0 mmol) was nitrated as per method B to produce **6d** as a white solid (130 mg, 69% yield), mp 240-243 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 13.03 (s, 1H), 9.35 (s, 1H), 8.37 (s, 1H). ESI-MS: *m/z* 198.0 $[M + H]^+$). HPLC flow rate 0.5 mL/min, t_R (acetonitrile/water 90:10) = 5.8 min, purity 96%.

3.3.13. 2-Ethyl-7-nitrothieno[3,2-*d*]pyrimidin-4(3*H*)-one (6e). Nitration of **7e** (180 mg, 1.0 mmol)) according to method B yielded **6e** as a white solid (158 mg, 70% yield), mp 270-273 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 12.87 (s, 1H), 9.31 (s, 1H), 2.70 (q, J = 5.7 Hz, 2H), 1.26 (t, J = 7.6 Hz, 3H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 163.29, 157.96, 149.31, 141.51, 138.89, 122.25, 28.28, 11.93. HRMS (*m/z*): $[M + H]^+$ calcd for $C_8H_8N_3O_3S$, 226.0281; found: 226.0289.

3.3.14. 7-Nitro-2-propylthieno[3,2-*d*]pyrimidin-4(3*H*)-one (6f). The nitro group was inserted in compound **7f** (194 mg, 1.0 mmol) according to method B to produce **6f** as a white solid (156 mg, 65% yield), mp 282-284 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 12.88 (s, 1H), 9.31 (s, 1H), 2.66 (t, J = 7.5 Hz, 2H), 1.80 – 1.70 (m, 2H), 0.95 (t, J = 7.3 Hz, 3H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 162.28, 157.95, 149.32, 141.50,

138.92, 122.21, 36.66, 20.95, 13.92. HRMS (*m/z*): $[M + H]^+$ calcd for $C_9H_{10}N_3O_3S$, 240.0437; found: 240.0448.

3.3.15. 7-Nitro-2-(3-nitrophenyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (6g). Nitro group was introduced onto **7g** (228 mg, 1.0 mmol) according to method B to obtain **6g** as a white solid (159 mg, 50% yield), mp >310 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 13.56 (s, 1H), 9.41 (s, 1H), 9.05 (t, *J* = 4.1 Hz, 1H), 8.62 (d, *J* = 8.0 Hz, 1H), 8.47 (d, *J* = 8.0 Hz, 1H), 7.90 (t, *J* = 7.8 Hz, 1H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 158.36, 155.04, 148.84, 148.44, 141.70, 139.77, 134.86, 134.14, 130.95, 126.81, 123.53, 123.47. HRMS (*m/z*): $[M + H]^+$ calcd for $C_{12}H_7N_4O_5S$, 319.0132; found: 319.0144.

3.3.16. 2-Methylthieno[3,2-*d*]pyrimidin-4(3*H*)-one (7a). Compound **7a** was prepared from methyl 3-aminothiophene-2-carboxylate **20** (157 mg, 1.0 mmol)) using method A as a white solid (83 mg, 50% yield), mp 235-238 °C [lit. mp 242 °C].[44] ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 12.41 (s, 1H), 8.14 (d, *J* = 8.2 Hz, 1H), 7.31 (d, *J* = 8.2 Hz, 1H), 2.37 (s, 3H). ESI-MS: *m/z* 167.0 $[M + H]^+$. HPLC flow rate 0.5 mL/min, t_R (acetonitrile/water 90:10) = 4.2 min, purity 98%.

3.3.17. 2-Methylfuro[3,2-*d*]pyrimidin-4(3*H*)-one (7b).[45] Intermediate **24b** (50 mg, 0.3 mmol) was refluxed in a mixture of 4M NaOH aqueous solution (10 mL) and methanol (20 mL) for 1 h. The resulting mixture was then neutralized to pH 7 using aqueous solution of 1M HCl and extracted with ethyl acetate three times. Organic layers were combined, dried over anhydrous $MgSO_4$ and concentrated. The residue was purified using flash chromatography by dry loading method to obtain **7b** as a white solid (16 mg, 35% yield), mp 228-231 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 12.46 (s, 1H), 8.17 (d, *J* = 8.2 Hz, 1H), 6.88 (d, *J* = 8.2 Hz, 1H), 2.34 (s, 3H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 155.46, 152.12, 150.32, 148.33, 136.16, 107.89, 20.98. HRMS (*m/z*): $[M + H]^+$ calcd for $C_7H_7N_2O_2$, 151.0502; found: 151.0487.

3.3.18. 2-Methylthieno[3,4-*d*]pyrimidin-4(3*H*)-one (7c).[46] Compound **7c** was prepared from **24c** (55 mg, 0.3 mmol) according the procedure described for the preparation of **7b**. Compound **7c** was obtained as a white solid (20 mg, 40% yield), mp 200-203 °C [lit. mp 232-233 °C].[46] ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 11.62 (s, 1H), 8.41 (d, J = 3.2 Hz, 1H), 7.63 (d, J = 3.3 Hz, 1H), 2.25 (s, 3H). ESI-MS: m/z 167.0 $[M + H]^+$. HPLC flow rate 0.5 mL/min, t_R (acetonitrile/water 90:10) = 4.4 min, purity 96%.

3.3.19. Thieno[3,2-*d*]pyrimidin-4(3*H*)-one (7d).[47, 48] To a round bottom flask containing 20 mL formamide, methyl 3-aminothiophene-2-carboxylate **20** (157 mg, 1.0 mmol) was added. The reaction mixture was stirred at rt for 6 h. The solution was then diluted with 100 mL of ethyl acetate, washed three times with brine and the organic layer was dried over $MgSO_4$. Compound **7d** was purified using flash chromatography by dry loading method as a white solid (99 mg, 65% yield), mp 222-224 °C [lit. mp 222-223 °C].[49] ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 12.51 (s, 1H), 8.19 (d, J = 5.4 Hz, 1H), 8.16 (s, 1H), 7.41 (d, J = 5.3 Hz, 1H). ESI-MS: m/z 153.0 $[M + H]^+$. HPLC flow rate 0.5 mL/min, t_R (acetonitrile/water 90:10) = 4.1 min, purity 97%.

3.3.20. 2-Ethylthieno[3,2-*d*]pyrimidin-4(3*H*)-one (7e).[44] To synthesize **7e**, intermediate **23e** (170 mg, 0.8 mmol) was treated with 20 mL of 30% ammonium hydroxide aqueous solution and stirred for 6 h at rt. Excess ammonia was released at low temperature and liquid was removed at high temperature under vacuum to obtain **7e** as a white solid (87 mg, 60% yield). ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 12.41 (s, 1H), 8.18 (d, J = 5.2 Hz, 1H), 7.30 (d, J = 2.6 Hz, 1H), 2.65 (t, J = 8.0 Hz, 2H), 0.97 (t, J = 7.8 Hz, 3H).

3.3.21. 2-Propylthieno[3,2-*d*]pyrimidin-4(3*H*)-one (7f).[39] Compound **7f** was prepared from **23f** (182 mg, 0.8 mmol) according to the procedure described for **7e** as a white

solid (90 mg, 58% yield). ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 12.39 (s, 1H), 8.14 (d, J = 5.2 Hz, 1H), 7.34 (d, J = 2.9 Hz, 1H), 2.60 (t, J = 7.6 Hz, 2H), 1.78 – 1.67 (m, 2H), 0.92 (t, J = 7.4 Hz, 3H).

3.3.22. 2-Phenylthieno[3,2-*d*]pyrimidin-4(3*H*)-one (7g).[50] Compound **7g** was prepared using **23g** (209 mg, 0.8 mmol) according to the procedure described for **7e**. Isolated product was a white solid (51 mg, 28% yield), mp 228-230 °C [lit. mp >240 °C].[50] ^{1}H NMR (400 MHz; D MSO-d_6; TMS) δ 12.41 (s, 1H), 8.12 (d, J = 5.42 Hz, 1H), 7.93 (d, J = 5.4 Hz, 2H,), 7.81 (d, J = 6.7 Hz, 1H), 7.68 – 7.58 (m, 3H). ESI-MS: m/z 229.0 $[M + H]^+$. HPLC flow rate 1 mL/min, t_R (acetonitrile/water 75:25) = 3.3 min, purity 95%.

3.3.23. 2,7-Dimethylthieno[3,2-*d*]pyrimidin-4(3*H*)-one (7h).[51] Compound **7h** was prepared according to method A from methyl 3-amino-4-methylthiophene-2-carboxylate **25** (171 mg, 1.0 mmol) as a white solid (77 mg, 43% yield), mp 255-257 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 12.39 (s, 1H), 7.78 (s, 1H), 2.39 (s, 3H), 2.28 (s, 3H). HRMS (m/z): $[M + H]^+$ calcd for $C_8H_9N_2OS$, 181.0430; found: 181.0442.

3.3.24. 7-Methylthieno[3,2-*d*]pyrimidin-4(3*H*)-one (7i).[43] Compound **7i** was prepared using methyl 3-amino-4-methylthiophene-2-carboxylate **25** (171 mg, 1.0 mmol) according to the procedure described for **7d** as a white solid (100 mg, 60% yield), mp 244-246 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 12.51 (s, 1H), 8.18 (s, 1H), 7.84 (s, 1H), 2.32 (s, 3H). ESI-MS: m/z 167.0 $[M + H]^+$. HPLC flow rate 1 mL/min, t_R (acetonitrile/water 65:35) = 6.2 min, purity 95%.

3.3.25. (*E*)-7-Nitro-2-styrylthieno[3,2-d]pyrimidin-4(3H)-one (8a). Compound **8a** was prepared using **6a** (150 mg, 0.7 mmol) and benzaldehyde (0.36 mL, 3.5 mmol) according to method C as a brown solid (60 mg, 28% yield), mp >310 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 13.00 (s, 1H), 9.35 (s, 1H), 7.98 (d, J = 16.0 Hz, 1H),

7.69 (d, *J* = 6.9 Hz, 2H), 7.50 – 7.43 (m, 3H), 7.07 (d, *J* = 16.1 Hz, 1H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 157.90, 155.90, 149.42, 141.60, 140.33, 139.33, 135.08, 130.64, 129.63, 128.33, 122.33, 120.66. HRMS (*m/z*): $[M + H]^+$ calcd for $C_{14}H_{10}N_3O_3S$, 300.0437; found: 300.0454.

3.3.26. (*E*)-2-(2-(Furan-2-yl)vinyl)-7-nitrothieno[3,2-*d*]pyrimidin-4(3*H*)-one (8b). Compound **8b** was prepared using **6a** (150 mg, 0.7 mmol) and furan-2-carbaldehyde (0.29 mL, 3.5 mmol) according to method C as a dark brown solid (45 mg, 22% yield), mp >310 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 12.99 (s, 1H), 9.34 (s, 1H), 7.90 (s, 1H), 7.81 (d, *J* = 15.5 Hz, 1H), 7.02 (d, *J* = 3.4 Hz, 1H), 6.82 (d, *J* = 15.5 Hz, 1H), 6.68 (q, *J* = 1.3 Hz, 1H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 157.87, 155.77, 151.27, 149.40, 146.30, 141.44, 139.28, 127.15, 121.92, 117.29, 116.09, 113.37. HRMS (*m/z*): $[M + H]^+$ calcd for $C_{12}H_8N_3O_4S$, 290.0230; found: 290.0253.

3.3.27. (*E*)-7-Nitro-2-(2-(thiophen-2-yl)vinyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (8c). Compound **8c** was prepared using **6a** (150 mg, 0.7 mmol) and 2-thiophene carbaldehyde (0.33 mL, 3.5 mmol) according to method C as a dark brown solid (66 mg, 31% yield), mp >310 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 12.90 (s, 1H), 9.34 (s, 1H), 8.14 (d, *J* = 15.4 Hz, 1H), 7.74 (d, *J* = 4.9 Hz, 1H), 7.56 (d, *J* = 3.3 Hz, 1H), 7.19 (t, *J* = 3.7 Hz, 1H), 6.80 (d, *J* = 15.3 Hz, 1H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 157.88, 155.71, 149.42, 141.49, 140.17, 139.33, 133.30, 132.21, 129.89, 129.24, 122.03, 118.94. HRMS (*m/z*): $[M + H]^+$ calcd for $C_{12}H_8N_3O_3S_2$, 306.0002; found: 306.0011.

3.3.28. (*E*)-4-(2-(7-Nitro-4-oxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-2-yl)vinyl)benzoic acid (8d). Compound **8d** was prepared using **6a** (150 mg, 0.7 mmol) and 4-formylbenzoic acid (525 mg, 3.5 mmol) according to method C as a dark brown solid (58 mg, 24% yield), mp >310 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 13.10

(s, 2H), 9.36 (s, 1H), 8.04 – 8.00 (m, 3H), 7.80 (d, *J* = 8.2 Hz, 2H), 7.18 (d, *J* = 16.3 Hz, 1H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 167.27, 157.88, 155.54, 149.30, 141.58, 139.42, 139.13, 139.00, 132.17, 130.48, 128.40, 122.99, 122.68. HRMS (*m/z*): $[M + H]^+$ calcd for $C_{13}H_{10}N_3O_5S$, 344.0336; found: 344.0349.

3.3.29. (*E*)-5-(2-(7-Nitro-4-oxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-2-yl)vinyl)furan-2-carboxylic acid (8e). Compound **8e** was prepared using **6a** (150 mg, 0.7 mmol) and 5-formyl-2-furoic acid (490 mg, 3.5 mmol) according to method C as a dark brown solid (47 mg, 20% yield), mp >310 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 13.43 (s, 1H), 13.03 (s, 1H), 9.36 (s, 1H), 7.83 (d, *J* = 15.8 Hz, 1H), 7.34 (d, *J* = 3.6 Hz, 1H), 7.16 (d, *J* = 3.6 Hz, 1H), 7.05 (d, *J* = 15.8 Hz, 1H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 159.46, 157.70, 155.13, 153.92, 149.19, 146.31, 141.47, 139.39, 126.38, 122.53, 120.98, 120.09, 116.79. HRMS (*m/z*): $[M + H]^+$ calcd for $C_{13}H_8N_3O_6S$, 334.0128; found: 334.0149.

3.3.30. (*E*)-2-(4-Fluorostyryl)-7-nitrothieno[3,2-*d*]pyrimidin-4(3*H*)-one (8f). Compound **8f** was prepared from **6a** (150 mg, 0.7 mmol) and 4-fluorobenzaldehyde (0.38 mL, 3.5 mmol) according to method C as a brown solid (44 mg, 20% yield), mp >310 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 13.00 (s, 1H), 9.34 (s, 1H), 7.96 (d, *J* = 16.0 Hz, 1H), 7.76 (dd, *J* = 10.2 Hz, 4.2 Hz, 2H), 7.32 (t, *J* = 8.9 Hz, 2H), 7.02 (d, *J* = 16.4 Hz, 1H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 163.50 (d, *J* = 247.8 Hz), 157.84, 155.77, 149.33, 141.47, 139.36, 138.97, 131.68 (d, *J* = 3.0 Hz), 130.52 (d, *J* = 8.2 Hz), 122.27, 120.45, 116.58 (d, *J* = 21.5 Hz). HRMS (*m/z*): $[M + H]^+$ calcd for $C_{14}H_9FN_3O_3S$, 318.0343; found: 318.0363.

3.3.31. (*E*)-2-(2-Fluorostyryl)-7-nitrothieno[3,2-*d*]pyrimidin-4(3*H*)-one (8g). Compound **8g** was prepared using **6a** (150 mg, 0.7 mmol) and 2-fluorobenzaldehyde (0.38 mL, 3.5 mmol) according to method C as a dark brown solid (82 mg, 37% yield),

mp 301-303 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 13.11 (s, 1H), 9.36 (s, 1H), 8.01 (d, J = 16.0 Hz, 1H), 7.80 (t, J = 3.7 Hz, 1H), 7.52 – 7.48 (m, 1H), 7.37 – 7.31 (m, 2H), 7.18 (d, J = 16.4 Hz, 1H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 161.03 (d, J = 249.4 Hz), 157.87, 155.64, 149.30, 141.58, 139.41, 132.53 (d, J = 3.4 Hz), 129.44, 129.41, 125.68 (d, J = 3.4 Hz), 123.19 (d, J = 6.4 Hz), 122.75, 122.72 (d, J = 27.7 Hz), 116.73 (d, J = 21.8 Hz). HRMS (*m/z*): $[M + H]^+$ calcd for $C_{14}H_9FN_3O_3S$, 318.0343; found: 318.0367.

3.3.32. (*E*)-2-(3-Fluorostyryl)-7-nitrothieno[3,2-*d*]pyrimidin-4(3*H*)-one (8h). Compound **8h** was prepared using **6a** (150 mg, 0.7 mmol) and 3-fluorobenzaldehyde (0.38 mL, 3.5 mmol) according to method C as a dark brown solid (73 mg, 33% yield), mp 304-307 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 13.03 (s, 1H), 9.36 (s, 1H), 7.96 (d, J = 16.2 Hz, 1H), 7.58 – 7.51 (m, 3H), 7.31 – 7.26 (m, 1H), 7.12 (d, J = 16.2 Hz, 1H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 162.99 (d, J = 244.1 Hz), 157.85, 155.59, 149.31, 141.60, 139.35, 138.88, 137.65 (d, J = 8.1 Hz), 131.57 (d, J = 8.3 Hz), 124.60 (d, J = 2.3 Hz), 122.61, 122.35, 117.27 (d, J = 21.4 Hz), 114.55 (d, J = 21.9 Hz). HRMS (*m/z*): $[M + H]^+$ calcd for $C_{14}H_9FN_3O_3S$, 318.0343; found: 318.0341.

3.3.33. (*E*)-7-Nitro-2-(4-nitrostyryl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (8i). Compound **8i** was obtained using **6a** (150 mg, 0.7 mmol) and 4-nitrobenzaldehyde (529 mg, 3.5 mmol) according to method C as a dark orange solid (48 mg, 20% yield), mp >310 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 13.11 (s, 1H), 9.36 (s, 1H), 8.30 (d, J = 8.8 Hz, 2H), 8.04 (d, J = 16.0 Hz, 1H), 7.96 (d, J = 8.6 Hz, 2H), 7.26 (d, J = 16.1 Hz, 1H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 157.80, 155.19, 149.18, 148.22, 141.60, 141.49, 139.49, 137.67, 129.36, 125.01, 124.70, 123.00. HRMS (*m/z*): $[M + H]^+$ calcd for $C_{14}H_9N_4O_5S$, 345.0288; found: 345.0333.

3.3.34. (*E*)-4-(2-(7-Nitro-4-oxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-2-yl)vinyl)benzonitrile (8j). Compound **8j** was prepared from **6a** (150 mg, 0.7 mmol) and 4-cyanobenzaldehyde (459 mg, 3.5 mmol) according to method C as a dark brown solid (70 mg, 31% yield), mp >310 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 13.08 (s, 1H), 9.36 (s, 1H), 8.00 (d, J = 8.4 Hz, 3H), 7.80 (d, J = 7.5 Hz, 2H), 7.17 (d, J = 15.7 Hz, 1H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 167.30, 157.99, 155.64, 149.29, 141.59, 139.38, 139.12, 138.95, 132.25, 130.47, 128.38, 123.09, 122.68. ESI-MS: m/z 325.0 $[M + H]^+$. HPLC flow rate 1 mL/min, t_R (acetonitrile/water 50:50) = 3.0 min, purity 95%.

3.3.35. (*E*)-2-(4-Chlorostyryl)-7-nitrothieno[3,2-*d*]pyrimidin-4(3*H*)-one (8k). Compound **8k** was prepared using **6a** (150 mg, 0.7 mmol) and 4-chlorobenzaldehyde (492 mg, 3.5 mmol) according to method C as a dark brown solid (47 mg, 20% yield), mp 307-308 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 13.02 (s, 1H), 9.35 (s, 1H), 7.95 (d, J = 15.9 Hz, 1H), 7.71 (d, J = 8.6 Hz, 2H), 7.54 (d, J = 8.5 Hz, 2H), 7.07 (d, J = 16.1 Hz, 1H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 157.94, 155.74, 149.35, 141.57, 139.36, 138.86, 135.06, 134.04, 130.02, 129.66, 122.48, 121.54. HRMS (m/z): $[M + H]^+$ calcd for $C_{14}H_9ClN_3O_3S$, 334.0048; found: 334.0072.

3.3.36. (*E*)-2-(4-Ethynylstyryl)-7-nitrothieno[3,2-*d*]pyrimidin-4(3*H*)-one (8l). Compound **8l** was prepared from **6a** (150 mg, 0.7 mmol) and 4-ethynylbenzaldehyde (455 mg, 3.5 mmol) according to method C as a dark brown solid (57 mg, 25% yield), mp >310 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 13.04 (s, 1H), 9.36 (s, 1H), 7.97 (d, J = 16.3 Hz, 1H), 7.70 (d, J = 8.3 Hz, 2H), 7.57 (d, J = 8.1 Hz, 2H), 7.10 (d, J = 16.0 Hz, 1H), 4.39 (s, 1H).). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 159.25, 157.89, 155.89, 149.41, 141.58, 140.31, 139.32, 138.94, 135.08, 130.63, 129.62, 128.32,

122.32, 120.65. HRMS (*m/z*): $[M + H]^+$ calcd for $C_{16}H_{10}N_3O_3S$, 324.0437; found: 324.0463.

3.3.37. (*E*)-2-(4-Hydroxystyryl)-7-nitrothieno[3,2-*d*]pyrimidin-4(3*H*)-one (8m). Compound **8m** was prepared using **6a** (150 mg, 0.7 mmol) and 4-hydroxybenzaldehyde (427 mg, 3.5 mmol) according to method C as a dark brown solid (62 mg, 28% yield), mp >310 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 12.90 (s, 1H), 10.09 (s, 1H), 9.34 (s, 1H), 7.91 (d, *J* = 15.7 Hz, 1H), 7.52 (d, *J* = 8.6 Hz, 2H), 6.87 – 6.82 (m, 3H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 160.14, 157.97, 156.46, 149.63, 141.53, 140.66, 139.25, 130.29, 126.14, 121.64, 116.76, 116.50. HRMS (*m/z*): $[M + H]^+$ calcd for $C_{14}H_{10}N_3O_4S$, 316.0387; found: 316.0402.

3.3.38. (*E*)-2-(4-Methoxystyryl)-7-nitrothieno[3,2-*d*]pyrimidin-4(3*H*)-one (8n). Compound **8n** was prepared using **6a** (150 mg, 0.7 mmol) and 4-methoxybenzaldehyde (0.43 mL, 3.5 mmol) according to method C as a dark brown solid (81 mg, 35% yield), mp 294-297 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 12.92 (s, 1H), 9.34 (s, 1H), 7.94 (d, *J* = 15.5 Hz, 1H), 7.64 (d, *J* = 9.0 Hz, 2H), 7.03 (d, *J* = 8.2 Hz, 2H), 6.92 (d, *J* =15.5 Hz, 1H), 3.82 (s, 3H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 161.41, 157.94, 156.28, 149.53, 141.53, 140.15, 139.21, 130.05, 127.68, 121.84, 117.93, 115.09, 55.82. HRMS (*m/z*): $[M + H]^+$ calcd for $C_{15}H_{12}N_3O_4S$, 330.0543; found: 330.0573.

3.3.39. 3-Benzyl-2-methyl-7-nitrothieno[3,2-*d*]pyrimidin-4(3*H*)-one (9a). Compound **9a** was prepared from the reaction of **6a** (106 mg, 0.5 mmol) and benzyl bromide (0.11 mL, 0.9 mmol) according to method D as a white solid (72 mg, 48% yield), mp 220-221 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 9.36 (s, 1H), 7.38 – 7.28 (m, 3H), 7.22 (d, *J* = 7.3 Hz, 2H), 5.43 (s, 2H), 2.56 (s, 3H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 160.06, 157.81, 147.49, 141.18, 139.77, 136.07, 129.31, 128.00,

126.89, 121.98, 47.03, 23.64. HRMS (*m/z*): $[M + H]^+$ calcd for $C_{14}H_{12}N_3O_3S$, 302.0594; found: 302.0616.

3.3.40. 2-Methyl-7-nitro-3-phenethylthieno[3,2-*d*]pyrimidin-4(3*H*)-one (9b). Compound **9b** was obtained from the reaction of **6a** (106 mg, 0.5 mmol) and (2-bromoethyl)benzene (0.12 mL, 0.9 mmol) according to method D as a white solid (66 mg, 42% yield), mp 227-229 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 9.33 (s, 1H), 7.35 – 7.24 (m, 5H), 4.28 (t, *J* = 7.8 Hz, 2H), 2.99 (t, *J* = 7.6 Hz, 2H), 2.56 (s, 3H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 159.82, 157.35, 147.31, 141.11, 139.52, 138.45, 129.30, 129.10, 127.19, 121.90, 46.37, 33.78, 23.37. HRMS (*m/z*): $[M + H]^+$ calcd for $C_{15}H_{14}N_3O_3S$, 316.0750; found: 316.0766.

3.3.41. 2-Methyl-7-nitro-3-(3-phenylpropyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (9c). Compound **9c** was obtained by reacting **6a** (106 mg, 0.5 mmol) with 1-bromo-3-phenylpropane (0.14 mL, 0.9 mmol) according to method D as a white solid (69 mg, 42% yield), mp 207-209 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 9.30 (s, 1H), 7.31 – 7.26 (m, 4H), 7.20 – 7.16 (m, 1H), 4.08 (t, *J* = 7.9 Hz, 2H), 2.71 (t, *J* = 7.6 Hz, 2H), 2.62 (s, 3H), 2.21 – 1.94 (m, 2H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 159.74, 157.41, 147.28, 141.33, 141.07, 139.35, 128.78, 128.72, 126.41, 121.86, 44.45, 32.77, 29.31, 23.32. HRMS (*m/z*): $[M + H]^+$ calcd for $C_{16}H_{16}N_3O_3S$, 330.0907; found: 330.0927.

3.3.42. 2-Methyl-7-nitro-3-(4-nitrobenzyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (9d). Compound **9d** was prepared by reacting **6a** (106 mg, 0.5 mmol) with 4-nitrobenzyl bromide (194 mg, 0.9 mmol) according to method D as a dark orange solid (104 mg, 60% yield), mp 218-220 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 9.38 (s, 1H), 8.21 (d, *J* = 8.7 Hz, 2H), 7.52 (d, *J* = 8.8 Hz, 2H), 5.55 (s, 2H), 2.56 (s, 3H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 159.94, 157.75, 147.57, 147.37, 143.91, 141.21, 139.86,

128.20, 124.39, 121.97, 46.96, 23.70. HRMS (*m/z*): $[M + H]^+$ calcd for $C_{14}H_{11}N_4O_5S$, 347.0445; found: 347.0456.

3.3.43. 2-Methyl-7-nitro-3-(2-nitrobenzyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (9e). Compound **9e** was obtained from the reaction of **6a** (106 mg, 0.5 mmol) and 2-nitrobenzyl bromide (194 mg, 0.9 mmol) according to method D as a white solid (95 mg, 55% yield), mp 190-192 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 9.38 (s, 1H), 8.23 (d, J = 8.2 Hz, 1H), 7.67 (t, J = 7.5 Hz, 1H), 7.61 (t, J = 8.0 Hz, 1H), 7.08 (d, J = 7.7 Hz, 1H), 5.71 (s, 2H), 2.54 (s, 3H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 160.09, 157.66, 147.87, 147.68, 141.23, 139.80, 135.17, 131.21, 129.31, 127.09, 125.98, 121.93, 45.54, 23.52. HRMS (*m/z*): $[M + H]^+$ calcd for $C_{14}H_{11}N_4O_5S$, 347.0445; found: 347.0468.

3.3.44. 2-Methyl-7-nitro-3-(3-nitrobenzyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (9f). Compound **9f** was prepared using **6a** (106 mg, 0.5 mmol) and 3-nitrobenzyl bromide (194 mg, 0.9 mmol) according to method D as a white solid (83 mg, 48% yield), mp 194-197 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 9.37 (s, 1H), 8.18 – 8.16 (m, 2H), 7.70 – 7.63 (m, 2H), 5.55 (s, 2H), 2.58 (s, 3H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 159.98, 157.88, 148.51, 147.56, 141.20, 139.85, 138.39, 133.62, 130.87, 123.05, 122.24, 122.00, 46.71, 23.78. HRMS (*m/z*): $[M + H]^+$ calcd for $C_{14}H_{11}N_4O_5S$, 347.0445; found: 347.0462.

3.3.45. 4-((2-Methyl-7-nitro-4-oxothieno[3,2-*d*]pyrimidin-3(4*H*)-yl)methyl)benzonitrile (9g). Compound **9g** was prepared from the reaction of **6a** (106 mg, 0.5 mmol) and 4-cyanobenzyl bromide (176 mg, 0.9 mmol) according to method D as a white solid (72 mg, 44% yield), mp 198-200 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 9.37 (s, 1H), 7.84 (d, J = 7.2 Hz, 2H), 7.44 (d, J = 7.5 Hz, 2H), 5.50 (s, 2H), 2.54 (s, 3H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 159.91, 157.74, 147.52, 141.82,

141.14, 139.86, 133.19, 127.88, 121.97, 119.09, 110.80, 47.05, 23.67. HRMS (*m/z*): $[M + H]^+$ calcd for $C_{15}H_{11}N_4O_3S$, 327.0546; found: 327.0562.

3.3.46. 3-(4-Methoxybenzyl)-2-methyl-7-nitrothieno[3,2-*d*]pyrimidin-4(3*H*)-one (9h). Compound **6a** (106 mg, 0.5 mmol) was reacted with 4-methoxybenzyl bromide (0.13 mL, 0.9 mmol) according to method D to obtain **9h** as a white solid (80 mg, 48% yield), mp 189-192 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 9.35 (s, 1H), 7.19 (d, J = 8.7 Hz, 2H), 6.91 (d, J = 8.7 Hz, 2H), 5.34 (s, 2H), 3.73 (s, 3H), 2.58 (s, 3H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 160.07, 159.09, 157.85, 147.41, 141.11, 139.78, 128.53, 127.85, 122.00, 114.66, 55.55, 46.53, 23.62. HRMS (*m/z*): $[M + H]^+$ calcd for $C_{15}H_{14}N_3O_4S$, 332.0700; found: 332.0714.

3.3.47. Ethyl 2-(2-methyl-7-nitro-4-oxothieno[3,2-*d*]pyrimidin-3(4*H*)-yl)acetate (10a). Compound **10a** was prepared by reacting **6a** (211 mg, 1.0 mmol) with ethyl bromoacetate (0.2 mL, 1.8 mmol) according to method D as a white solid (134 mg, 45% yield), mp 145-147 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 9.36 (s, 1H), 5.00 (s, 2H), 4.20 (q, J = 7.3 Hz, 2H), 2.61 (s, 3H), 1.23 (t, J = 7.2 Hz, 3H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 167.96, 159.86, 157.22, 147.55, 141.22, 140.01, 121.31, 62.14, 46.06, 23.05, 14.44. HRMS (*m/z*): $[M + H]^+$ calcd for $C_{11}H_{12}N_3O_5S$, 298.0492; found: 298.0498.

3.3.48. tert-Butyl 2-(2-methyl-7-nitro-4-oxothieno[3,2-*d*]pyrimidin-3(4*H*)-yl)acetate (10b). Compound **6a** (211 mg, 1.0 mmol) was reacted with *tert*-butyl 2-chloroacetate (0.26 mL, 1.8 mmol) to obtain **10b** as a white solid (195 mg, 60% yield), mp 190-193 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 9.36 (s, 1H), 4.91 (s, 2H), 2.58 (s, 3H), 1.44 (s, 9H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ167.02, 159.81, 157.21, 147.51, 141.20, 139.98, 121.34, 83.08, 46.50, 28.06, 23.46. HRMS (*m/z*): $[M + H]^+$ calcd for $C_{13}H_{16}N_3O_5S$, 326.0805; found: 326.0804.

3.3.49. 2-(2-Methyl-7-nitro-4-oxothieno[3,2-*d*]pyrimidin-3(4*H*)-yl)acetic acid (10c). Compound **10a** (100 mg, 0.3 mmol) was hydrolyzed to obtain **10c** (52 mg, 65% yield) as a white solid by following procedure described for preparation of **27**, mp 225-228 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 13.52 (s, 1H), 9.35 (s, 1H), 4.92 (s, 2H), 2.60 (s, 3H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 169.31, 159.93, 157.24, 147.51, 141.19, 139.88, 121.38, 45.96, 23.48. HRMS (*m*/*z*): $[M + H]^+$ calcd for $C_9H_8N_3O_5S$, 270.0179; found: 270.0173.

3.3.50. 2-(2-Methyl-7-nitro-4-oxothieno[3,2-*d*]pyrimidin-3(4*H*)-yl)-*N*-phenylacetamide (10d).[52] A mixture of compound **10c** (269 mg, 1.0 mmol), EDC (211 mg, 1.1 mmol), HBTU (417 mg, 1.1 mmol) and triethylamine (0.28 mL, 2.0 mmol) in dichloromethane was stirred for 30 min, then aniline (0.15 mL, 1.5 mmol) was added. Upon complete conversion of starting material, the reaction mixture was diluted with water and subsequently extracted with ethyl acetate. Combined organic layers were dried over $MgSO_4$ and evaporated. Crude product was purified using dry loading method and dichloromethane/methanol mobile phase on flash chromatography to obtain **10d** as a light yellow solid (121 mg, 35% yield), mp 260-262 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 10.51 (s, 1H), 9.36 (s, 1H), 7.58 (d, J = 7.4 Hz, 2H), 7.33 (t, J = 7.4 Hz, 2H), 7.08 (t, J = 4.3 Hz, 1H), 5.04 (s, 2H), 2.63 (s, 3H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 165.22, 160.51, 157.42, 147.57, 141.20, 139.88, 138.95, 129.38, 124.18, 121.41, 119.59, 47.55, 23.72. HRMS (*m*/*z*): $[M + H]^+$ calcd for $C_{15}H_{13}N_4O_4S$, 345.0652; found: 345.0626.

3.3.51. 2-Methyl-3-(2-morpholino-2-oxoethyl)-7-nitrothieno[3,2-*d*]pyrimidin-4(3*H*)-one (10e). Compound **10e** was prepared as per the procedure described for **10d** using compound **10c** (269 mg, 1.0 mmol) and morpholine (0.13 mL, 1.5 mmol) as starting materials. Purified product was obtained as a white solid (112 mg, 33% yield),

mp 239-241 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 9.35 (s, 1H), 5.15 (s, 2H), 3.68 (t, J = 3.3 Hz, 2H), 3.60 (t, J = 4.6 Hz, 4H), 3.47 (t, J = 4.1 Hz, 2H), 2.53 (s, 3H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 164.81, 160.42, 157.29, 147.52, 141.20, 139.69, 121.39, 66.51, 45.49, 45.30, 23.50. HRMS (*m*/*z*): $[M + H]^+$ calcd for $C_{13}H_{15}N_4O_5S$, 339.0758; found: 339.0739.

3.3.52. 2-Methyl-3-(2-(4-methylpiperazin-1-yl)-2-oxoethyl)-7-nitrothieno[3,2-*d*]pyrimidin-4(3*H*)-one (10f). Compound **10f** was prepared as per the procedure described for **10d**, using compound **10c** (269 mg, 1.0 mmol) and *N*-methyl piperazine (0.17 mL, 1.5 mmol) as starting materials. Purified product was obtained as a white solid (141 mg, 40% yield), mp 140-143 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 9.34 (s, 1H), 5.14 (s, 2H), 3.57 (t, J = 4.7 Hz, 2H), 3.48 (t, J = 5.0 Hz, 2H), 2.52 (s, 3H), 2.41 (t, J = 4.7 Hz, 2H), 2.30 (t, J = 4.7 Hz, 2H), 2.22 (s, 3H). HRMS (*m*/*z*): $[M + H]^+$ calcd for $C_{14}H_{18}N_5O_4S$, 352.1074; found: 352.1074.

3.3.53. 4-Chloro-2-methyl-7-nitrothieno[3,2-*d*]pyrimidine (11a).[53] Compound **6a** (211 mg, 1.0 mmol) was added into a round bottom flask containing phosphorus oxychloride (20 mL, 215 mmol). Reaction mixture was stirred under reflux for 18 h. Once starting material was fully converted, the solution was transferred portion wise into ice water. The mixture was neutralized with aqueous solution of $NaHCO_3$ and extracted 3 times with ethyl acetate. Organic layers were combined, dried over anhydrous $MgSO_4$ and evaporated. Resulting crude product was purified by flash chromatography (dichloromethane/methanol 93:3) to obtain a pale yellow solid (92 mg, 40% yield), mp 164-165 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 9.69 (s, 1H), 2.81 (s, 3H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 156.32, 152.18, 147.02, 140.67, 140.63, 135.54, 115.89. HRMS (*m*/*z*): $[M + H]^+$ calcd for $C_7H_3ClN_3O_2S$, 229.9786; found: 229.9765.

3.3.54. 2-Methyl-7-nitro-*N*-phenylthieno[3,2-*d*]pyrimidin-4-amine (11b).[53]

Compound **11a** (50 mg, 0.2 mmol) and aniline (0.09 mL, 1.0 mmol) were added into a microwave tube. The mixture was kept in a single cavity microwave initiator, and the reaction was carried out at 150 °C for 50 min. The reaction mass was diluted with a mixture of ethyl acetate/water. Organic layer was separated, dried over $MgSO_4$, and evaporated. Resulting crude product was purified by flash chromatography (dichloromethane/methanol 99:1) to obtain **11b** as a white solid (20 mg, 35% yield), mp 202-205 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 10.01 (s, 1H), 9.36 (s, 1H), 7.78 (d, J = 7.7 Hz, 2H), 7.41 (t, J = 7.7 Hz, 2H), 7.17 (t, J = 7.7 Hz, 1H), 2.57 (s, 3H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 165.72, 155.54, 151.79, 141.17, 139.10, 138.91, 129.19, 124.76, 123.11, 113.89, 26.31. HRMS (*m/z*): $[M + H]^+$ calcd for $C_{13}H_{11}N_4O_2S$, 287.0597; found: 287.0621.

3.3.55. 4-(2-Methyl-7-nitrothieno[3,2-*d*]pyrimidin-4-yl)morpholine (11c). Compound **11c** was prepared as per the procedure described for **11b**, using **11a** (50 mg, 0.2 mmol) and morpholine (0.09 mL, 1.0 mmol) as starting materials. Target compound was isolated as a pale yellow solid (25 mg, 45% yield), mp 182-185 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 9.40 (s, 1H), 3.93 (t, J = 5.1 Hz, 4H), 3.76 (t, J = 5.1 Hz, 4H), 2.53 (s, 3H). HRMS (*m/z*): $[M + H]^+$ calcd for $C_{11}H_{13}N_4O_3S$, 281.0703; found: 281.0724.

3.3.56. (*E*)-3-Benzyl-2-(4-fluorostyryl)-7-nitrothieno[3,2-*d*]pyrimidin-4(3*H*)-one (12a). Reaction of **9a** (100 mg, 0.3 mmol) and 4-fluorobenzaldehyde (0.16 mL, 1.5 mmol) was carried out according to method C to obtain **12a** as a brown solid (27 mg, 22% yield), mp 192-195 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 9.40 (s, 1H), 7.93 (d, J = 15.1 Hz, 1H), 7.78 (q, J = 3.4 Hz, 2H), 7.40 – 7.22 (m, 8H), 5.70 (s, 2H). HRMS (*m/z*): $[M + H]^+$ calcd for $C_{21}H_{15}FN_3O_3S$, 408.0813; found: 408.0815.

3.3.57. (*E*)-4-Chloro-2-(4-fluorostyryl)-7-nitrothieno[3,2-*d*]pyrimidine (12b). Compound **8f** (100 mg, 0.3 mmol) was reacted with phosphorous oxychloride (15 mL, 161 mmol) to afford **12b** using procedure described for the preparation of **11a** as a yellow solid (72 mg, 72% yield), mp 256-258 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 9.69 (s, 1H), 8.06 (d, J = 16.1 Hz, 1H), 7.95 – 7.91 (m, 2H), 7.42 (d, J = 16.0 Hz, 1H), 7.28 (t, J = 8.9 Hz, 2H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 162.34 (d, J = 247.8 Hz), 162.08, 153.90, 151.96, 143.35, 140.01, 138.23, 131.15 (d, J = 2.9 Hz), 129.81 (d, J = 8.6 Hz), 126.55, 125.14, 115.33 (d, J = 21.7 Hz). HRMS (*m/z*): $[M + H]^+$ calcd for $C_{14}H_8ClFN_3O_2S$, 336.0004; found: 335.9991.

3.3.58. (*E*)-4-(2-(4-fluorostyryl)-7-nitrothieno[3,2-*d*]pyrimidin-4-yl)morpholine (12c). Compound **12b** (67 mg, 0.2 mmol) and morpholine (0.09 mL, 1.0 mmol) were reacted according to the procedure described for **11b** to afford **12c** as a white solid (25 mg, 32% yield), mp 245-248 °C. ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 9.43 (s, 1H), 7.93 (d, J = 16.3 Hz, 1H), 7.82 (dd, J = 8.3 Hz, 3.2 Hz, 2H), 7.25 (t, J = 8.3 Hz, 2H), 7.15 (d, J = 7.8 Hz, 1H), 4.02 (t, J = 8.9 Hz, 4H), 3.81 (t, J = 3.8 Hz, 4H). ^{13}C NMR (100 MHz; DMSO-d_6; TMS): δ 162.98 (d, J = 247.3 Hz), 161.71, 157.63, 152.85, 141.60, 137.98, 136.81, 132.77 (d, J = 3.3 Hz), 130.28 (d, J = 8.2 Hz), 128.08, 116.25 (d, J = 21.6 Hz), 113.05, 66.33, 46.30. HRMS (*m/z*): $[M + H]^+$ calcd for $C_{18}H_{16}FN_4O_3S$, 387.0922; found: 387.0938.

3.3.59. 2-Methyl-8-nitro-4*H*-benzo[*d*][1,3]oxazin-4-one (16). Intermediate **16** was prepared using commercially available methyl 2-amino-3-nitrobenzoate **13** (196 mg, 1.0 mmol) and acetic anhydride according to method A as an orange solid (180 mg, 87% yield). ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 8.12 (d, J = 7.9 Hz, 1H), 8.08 (d, J = 8.0 Hz, 1H), 7.49 (t, J = 7.9 Hz, 1H), 2.05 (s, 3H).

3.3.60. 2-Methyl-4*H*-pyrido[2,3-*d*][1,3]oxazin-4-one (17).[54] Intermediate **17** was prepared using commercially available methyl 2-aminonicotinate **14** (152 mg, 1.0 mmol) and acetic anhydride according to method A as a white solid (105 mg, 65% yield). ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 8.22 (dd, J = 4.9 Hz, 1.7 Hz, 1H), 8.15 (dd, J = 7.8 Hz, 1.8 Hz, 1H), 6,70 (dd, J = 7.7 Hz, 4.9 Hz, 1H), 3.83 (s, 3H).

3.3.61. 8-Fluoro-2-methyl-4*H*-benzo[*d*][1,3]oxazin-4-one (18). Intermediate **18** was prepared using commercially available methyl 2-amino-3-fluorobenzoate **15** (169 mg, 1.0 mmol) and acetic anhydride according to method A as a white solid (143 mg, 80% yield). ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 7.93 (d, J = 7.7 Hz, 1H), 7.81 (d, J = 8.0 Hz, 1H), 7.58 (t, J = 7.9 Hz, 1H), 2.43 (s, 3H).

3.3.62. Methyl 3-acetamidothiophene-2-carboxylate (22a). Intermediate **22a** was prepared using commercially available methyl 3-aminothiophene-2-carboxylate **19** (157 mg, 1.0 mmol) and acetic anhydride (20 mL) according to method A as a white solid (167 mg, 84% yield). ^{1}H NMR (400 MHz; $CDCl_3$; TMS) δ 9.99 (s, 1H), 7.93 (d, J = 5.4 Hz, 1H), 7.89 (d, J = 5.4 Hz, 1H), 3.84 (s, 3H), 2.17 (s, 3H).

3.3.63. Methyl 3-acetamidofuran-2-carboxylate (22b).[55] Intermediate **22b** was prepared using commercially available methyl 3-aminofuran-2-carboxylate **20** (141 mg, 1.0 mmol) and acetic anhydride (20 mL) according to method A as a white solid (119 mg, 65% yield). ^{1}H NMR (400 MHz; $CDCl_3$; TMS) δ 12.66 (s, 1H), 7.85 (s, 1H), 6.82 (s, 1H), 2.64 (s, 3H), 2.33 (s, 3H).

3.3.64. Methyl 4-acetamidothiophene-3-carboxylate (22c). Intermediate **22c** was prepared using commercially available methyl 4-aminothiophene-3-carboxylate **21** (157 mg, 1.0 mmol) and acetic anhydride (20 mL) according to method A as a white solid (165 mg, 83% yield). ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 9.83 (s, 1H), 8.35 (d, J = 3.5 Hz, 1H), 7.90 (d, J = 3.6 Hz, 1H), 3.85 (s, 3H), 2.13 (s, 3H).

3.3.65. Methyl 3-propionamidothiophene-2-carboxylate (22e).[56] Methyl 3-aminothiophene-2-carboxylate **19** (157 mg, 1.0 mmol) was stirred with triethylamine (0.21 mL, 1.5 mmol) and propionyl bromide (0.11 mL, 1.2 mmol) in dichloromethane at room temperature for 4 h. The mixture was then neutralized with 1 molar aqueous HCl solution and extracted with dichloromethane three times. Combined organic layers were dried over anhydrous $MgSO_4$ and concentrated under vacuum. Product was isolated as a white solid (171 mg, 80% yield). ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 10.02 (s, 1H), 7.97 (d, J = 5.4 Hz, 1H), 7.90 (d, J = 5.4 Hz, 1H), 3.84 (s, 3H), 2.46 (t, J = 7.5 Hz, 2H), 1.11 (t, J = 7.5 Hz, 3H).

3.3.66. Methyl 3-butyramidothiophene-2-carboxylate (22f). Intermediate **22f** was prepared using methyl 3-aminothiophene-2-carboxylate **19** (157 mg, 1.0 mmol) and butanoyl bromide (0.12 mL, 1.2 mmol) as starting materials following procedure described for the preparation of **22e**. Isolated product was white solid (193 mg, 85% yield). ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 10.11 (s, 1H), 8.03 (d, J = 5.3 Hz, 1H), 7.89 (d, J = 5.3 Hz, 1H), 3.83 (s, 3H), 2.76 (t, J = 7.4 Hz, 2H), 1.51 – 1.37 (m, 2H), 0.91 (t, J = 7.4 Hz, 3H).

3.3.67. Methyl 3-benzamidothiophene-2-carboxylate (22g). Intermediate **22g** was prepared using methyl 3-aminothiophene-2-carboxylate **19** (157 mg, 1.0 mmol) and benzoyl bromide (0.14 mL, 1.2 mmol) as starting materials by procedure described for the preparation of **22e**. Isolated product was white solid (214 mg, 82% yield). ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 11.02 (s, 1H), 8.12 (d, J = 5.3 Hz, 1H), 8.00 (d, J = 5.3 Hz, 1H,), 7.96 (d, J = 7.1 Hz, 2H), 7.71 – 7.61 (m, 3H), 3.89 (s, 3H).

3.3.68. Methyl 3-acetamido-4-methylthiophene-2-carboxylate (22h). Intermediate **22h** was prepared using commercially available methyl 3-amino-4-methylthiophene-2-carboxylate **24** (171 mg, 1.0 mmol) and acetic anhydride as starting materials according

to method A as a white solid (160 mg, 75% yield). ^{1}H NMR (400 MHz; $CDCl_3$; TMS) δ 9.62 (s, 1H), 7.51 (s, 1H), 3.75 (s, 3H), 2.03 - 2.02 (m, 6H).

3.3.69. 3-Acetamidofuran-2-carboxamide (23b).[57] Intermediate **23b** was prepared using **22b** (168 mg, 1.0 mmol) and ammonium hydroxide aqueous solution (30 mL) according to method A as a white solid (103 mg, 62% yield). ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 9.66 (s, 1H), 7.77 (s, 1H), 7.70 (d, J = 1.7 Hz, 1H), 7.55 (s, 1H), 7.22 (d, J = 1.7 Hz, 1H), 2.10 (s, 3H).

3.3.70. 4-Acetamidothiophene-3-carboxamide (23c).[46] Intermediate **23c** was prepared using **22c** (199 mg, 1.0 mmol) and ammonium hydroxide aqueous solution (30 mL) according to method A as a white solid (74 mg, 40% yield). ^{1}H NMR (400 MHz; DMSO-d_6; TMS) δ 11.00 (s, 1H), 8.27 (d, J = 3.4 Hz, 1H), 8.14 (s, 1H), 7.87 (d, J = 3.3 Hz, 1H), 7.61 (s, 1H), 2.08 (s, 3H).

3.4. Minimum Inhibitory Concentrations (MICs) of Thienopyrimidinone Analogues against *C. difficile* Strains

Following the guidelines defined by the Clinical and Laboratory Standards Institute (CLSI),[58] *Clostridium difficile* strains were grown anaerobically on brain heart infusion supplemented (BHIS) agar plates (Brain heart infusion, BD, supplemented with yeast extract, Vitamin K1 and Hemin, Sigma) at 37 °C for 48 h. Afterwards, a bacterial suspension of ~10^5 CFU/mL was prepared in BHIS broth and seeded in 96-well plates containing serial dilutions of the compounds and controls. Plates were then incubated anaerobically at 37 °C for 48 h. Reported MICs are the minimum concentration of each compound at which inhibition of the bacterial growth could be visually observed.[59] The MBC of the most potent compound, **8f**, was determined by subculturing **8f**-inhibited bacteria on a drug-free BHIS agar plates and incubated anaerobically at 37 °C for 24 h.

Reported MBC is the concentration at which 99.9% of the initial bacterial count was eradicated.[60, 61]

3.5. Time-kill assay of 8f against *C. difficile*

An 18-20 h culture of *C. difficile* ATCC BAA 1870 was diluted 1:50 in fresh BHIS broth to achieve a starting concentration of 10^6 CFU/mL. The bacterial suspension was mixed with 8 X MIC of **8f**, vancomycin, fidaxomicin or DMSO in triplicates. Bacterial concentration was measured at the indicated time points by serially diluting samples from each bacterial suspension followed by culturing, in duplicates on BHIS agar plates. CFU were counted after anaerobic incubation for 24 h at 37 °C.[62]

3.6. In Vitro Antimicrobial Evaluation of Thienopyrimidinone Analogues against Normal Microflora

With slight modification, CLSI and previous reports were followed in order to determine the MICs of the most active compounds against human microflora.[63, 64] Bacteria were first grown for 48 hours at 37 °C, anaerobically using BHIS agar for *Bifidobacterium* and in 5% CO_2 using MRS agar plate for *Lactobacillus*. Approximately a 10^5 CFU/mL suspension was prepared (in BHIS broth for *Bifidobacterium* or in MRS broth for *Lactobacillus*) for each strain and seeded in 96-well plates. Compounds were added at the required concentrations in the 96-well plates and incubated as mentioned for each species for 48 hours at 37 °C before recording the MIC values.

For *Escherichia coli* and *Enterobacter cloacae*, the activity of the compounds was tested in accordance with the CLSI.[63] Briefly, bacteria were grown on tryptic soy agar (TSA) plates for 16-20 h at 37 °C. A bacterial suspension was prepared in phosphate buffered saline (PBS), matched to the turbidity of a 0.5 McFarland standard solution and diluted in tryptic soy broth (TSB) to achieve a bacterial concentration of ~ 10^5 CFU/mL. The final bacterial suspension was incubated in 96-well plates with serial

dilutions of the compounds and the controls for 16-20 h at 37 °C. MICs were defined as the lowest concentration of each agent that inhibited the bacterial growth.[65,66]

3.7. In Vitro Cytotoxicity Analysis of Thienopyrimidinone Analogues

The most potent compounds were selected for further testing for their cytotoxicity against three different cell lines; human colon colorectal adenocarcinoma (Caco-2), human ileocecal adenocarcinoma (HRT-18) and African green monkey kidney cells (Vero) as described previously.[67,68] Briefly, cells were grown in T75 flasks at 37 °C in 5% CO_2 atmosphere till they reached ~90% confluency using the growth media recommended by the supplier. Cells were transferred to cell culture-treated 96-well plates, incubated at 37 °C in 5% CO_2 and allowed to reach confluency. Next, the growth media were replaced with fresh ones containing the indicated concentrations of the compounds or DMSO (as a negative control) in triplicates and incubated at 37 °C in 5% CO_2 for 2 h. After incubation, media were removed, and the cells were washed before the addition of 20% MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium). Added MTS was incubated with the cells for additional 4 h at 37 °C in 5% CO_2,then the absorbance for each well was recorded as an optical density at 490 nm. Data is presented as percentage cell viability as compared to the DMSO treated cells.

3.8. Determination of the Aqueous Solubility of Thienopyrimidinone Analogues

Solution at a concentration of 1 mg/mL was obtained by dissolving interested compound in methanol. The stock solution was passed through a 0.45-micron nylon membrane filter. Samples at different concentrations (1 μg/mL, 5 μg/mL, 50 μg/mL, and 100 μg/mL) were prepared and loaded onto HPLC. Isocratic mobile phase (acetonitrile/water 50:50) was used and a flow rate as 1.0 mL/min. Standard curve was achieved by plotting AUC (area under the curve) versus concentration at 254 nm. To

prepare saturated solution, 3 mg of target compound was added into an Eppendorf tube containing 3 mL PBS solution. The mixture was agitated for 24 h at 25 °C and centrifuged for 3 min at 16000 rpm. A mixture of 300 μL of supernatant and 300 μL acetonitrile was prepared. Absorbance was measured on HPLC, and solubility was calculated from absorbance, standard curve and dilution factor.

3.9. Assessment of the Stability of Thienopyrimidinone Analogues in Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF)

Stability of target compounds in SGF (pH = 1.6) and SIF (pH = 6.0) was evaluated following reported procedure with modification. Stock solutions at concentration of 50 μg/mL were prepared with methanol. Mixture of 200 μL of stock solution and 800 μL SGF/SIF was stirred vigorously and incubated at 37 °C. After 4 h and 8 h incubation, samples were loaded onto HPLC and eluted using isocratic mobile phase (acetonitrile/water 50:50) at a flow rate of 1.0 mL/min. The remaining percentage at each injection time point was calculated as AUC (after incubation)/AUC (before incubation) at λ 254 nm.[69]

3.10. In Silico PAINS Analysis

All the synthesized target compounds were subjected to PAINS filters by using a KNIME (v3.74, KNIME GmbH, Konstanz, Germany) workflow.[70] Molecular formula strings of target compounds were manually input into the workflow, and the output file for the run indicated no PAINS were found.

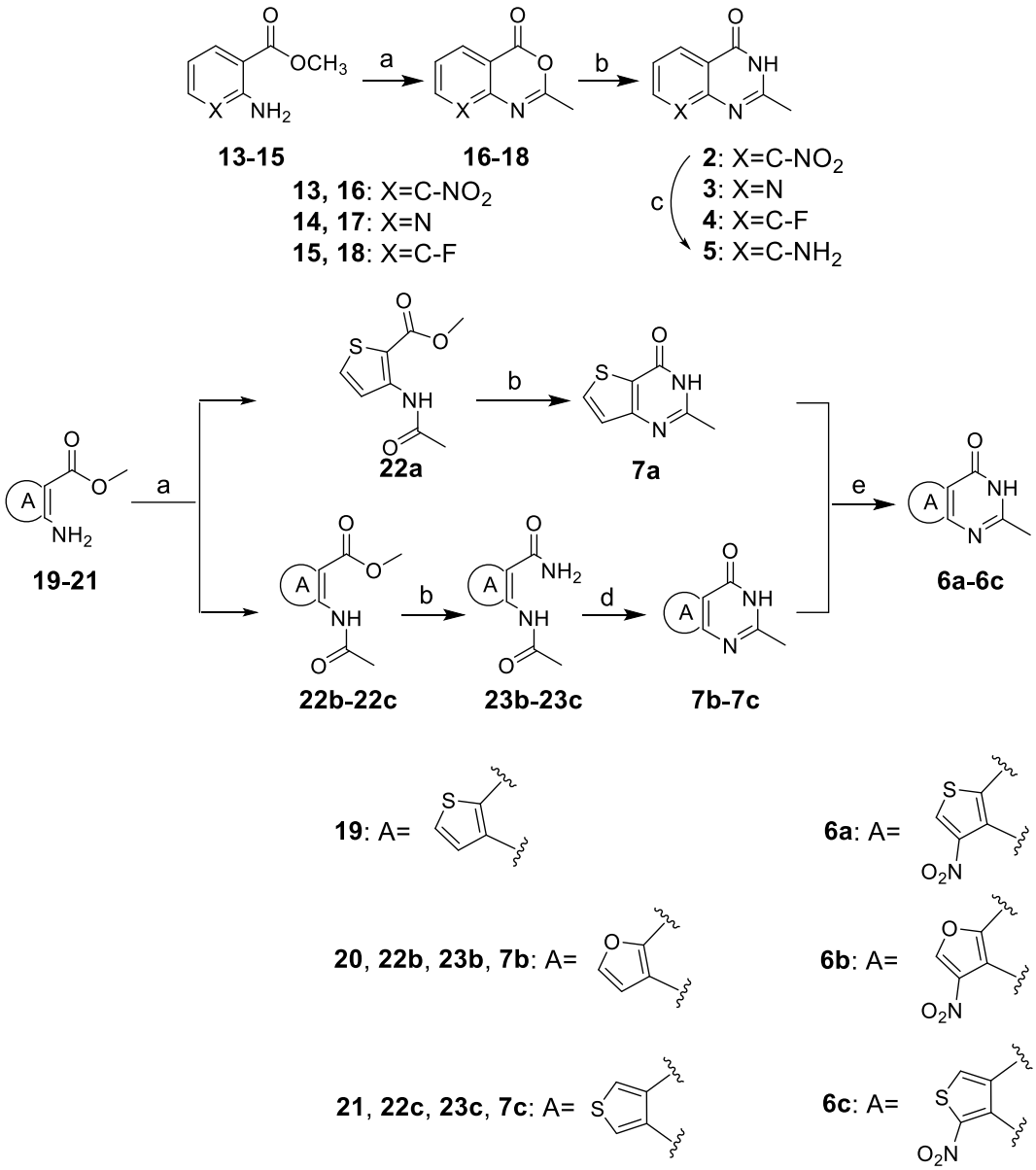
Chapter IV. Results and Discussion

4.1. Chemistry

To pursue more potent C. difficile inhibitor by exploring chemical space around thieno[3,2-*d*]pyrimidin-4(3*H*)-one scaffold, target compounds were obtained according to synthetic routes described in schemes 1- 6.

4.1.1. Synthesis of Fused Pyrimidinone Derivatives

Fused pyrimidinone derivatives were synthesized starting from amino and methyl ester substituted aromatic/heteroaromatic intermediates (Scheme 1). Acetylation of commercially available chemicals **13**-**15** and **19**-**21** was performed using acetic anhydride, and subsequent treatment of intermediates with ammonium hydroxide to obtain compounds **2**-**4** and **7a**.[34] Hydrogenation of the nitro group of **2** yielded **5**.[42] The reaction conditions used for the preparation of **7b** and **7c** required an alternate strategy due to varying reactivity of starting materials. As illustrated, intermediates **23b** and **23c** were prepared according to the procedure used for the synthesis of compound **2** and heated in a mixture of NaOH, water and methanol to obtain the cyclized compounds **7b** and **7c**.[45] The nitro group was introduced on **7a**-**7c** by treating with fuming nitric acid and concentrated sulfuric acid mixture to obtain nitro derivatives **6a**-**6c**.[71]

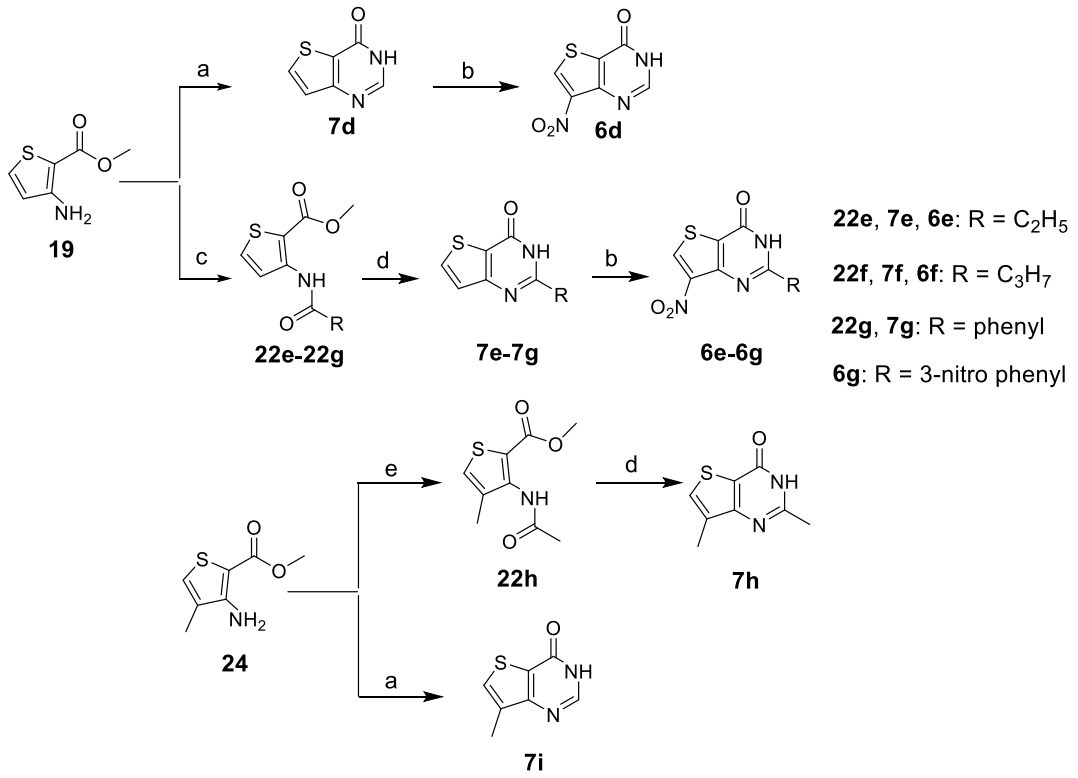


Scheme 1. Reagents and conditions: (a) acetic anhydride, **13** (for **16**), **14** (for **17**), **15** (for **18**), **19** (for **22a**), **20** (for **22b**), **21** (for **22c**), rt, 12-24 h, 65-85%; (b) 30% NH_4OH, **16** (for **2**), **17** (for **3**), **18** (for **4**), **22a** (for **7a**), **22b** (for **23b**), **22c** (for **23c**), rt, 6-8 h, 62-70%; (c) H_2/Pd/C, MeOH, 50 psi, rt, 8 h, 70%; (d) Aq. NaOH, MeOH, **23b** (for **7b**), **23c** (for **7c**), reflux, 4-6 h, 35-40%; (e) HNO_3, conc. H_2SO_4, 0 °C - rt, 4-12 h, 40-82%.

4.1.2. Synthesis of Thienopyrimidinone Derivatives

To investigate the role of *C*2-methyl group, **7d**-**7g** with *C*2-H, -ethyl, -propyl, and -phenyl substituents were prepared from **19** and **22e**-**22g**, respectively.[47,72] The nitrated derivatives **6d**-**6f** were prepared from **7d**-**7f** by following the same method used for the preparation of **6a**.[71] Target compound **6g** was obtained from nitration of **7g** wherein two

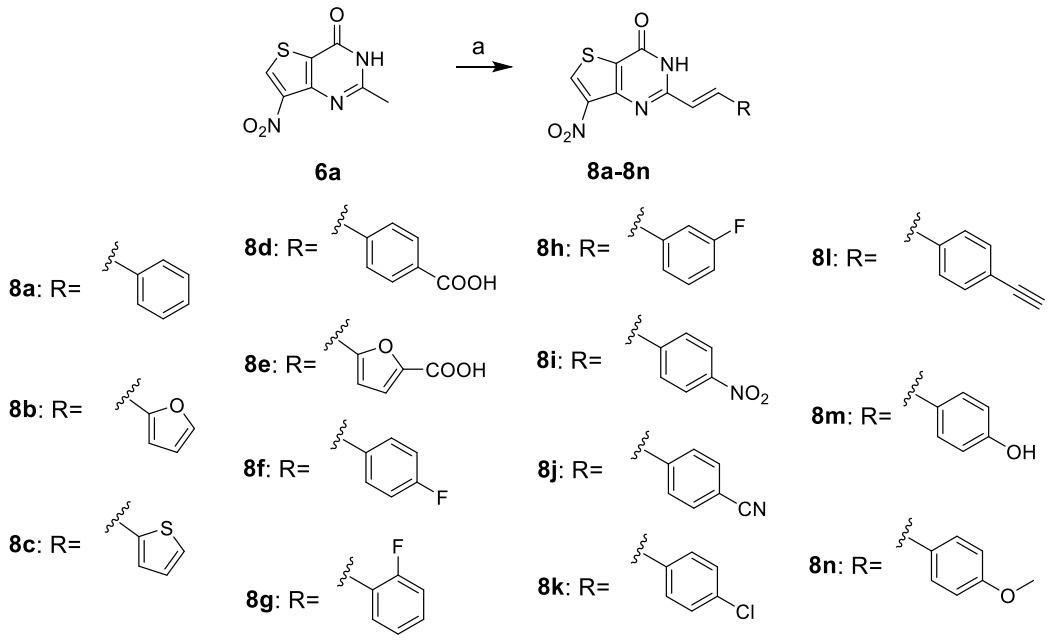
nitro groups (*C*7 of the bicyclic scaffold and *meta*-position of the *C*2-phenyl substituent) were introduced in the same reaction. To analyze the role of *C*7-nitro group, compounds with *C*7-methyl group (**7h** and **7i**) were synthesized from commercially available **24** over two-steps for **7h** and one-step for **7i** (Scheme 2).



Scheme 2. Reagents and conditions: (a) formamide, rt, 6-8 h, 60-65%; (b) HNO_3, conc. H_2SO_4, **7e** (for **6e**), **7f** (for **6f**), **7g** (for **6g**), 0 °C - rt, 6-8 h, 50-70%. (c) triethyl amine, DCM, propionyl bromide (for **22e**), butanoyl bromide (for **22f**), benzoyl chloride (for **22g**), rt, 4-8 h, 80-85%; (d) 30% NH_4OH, **22e** (for **7e**), **22f** (for **7f**), **22g** (for **7g**), **22h** (for **7h**), rt, 6-8 h, 28-60%; (e) acetic anhydride, rt, 14 h, 75%.

4.1.3. Synthesis of *C*2-Styryl Derivatives

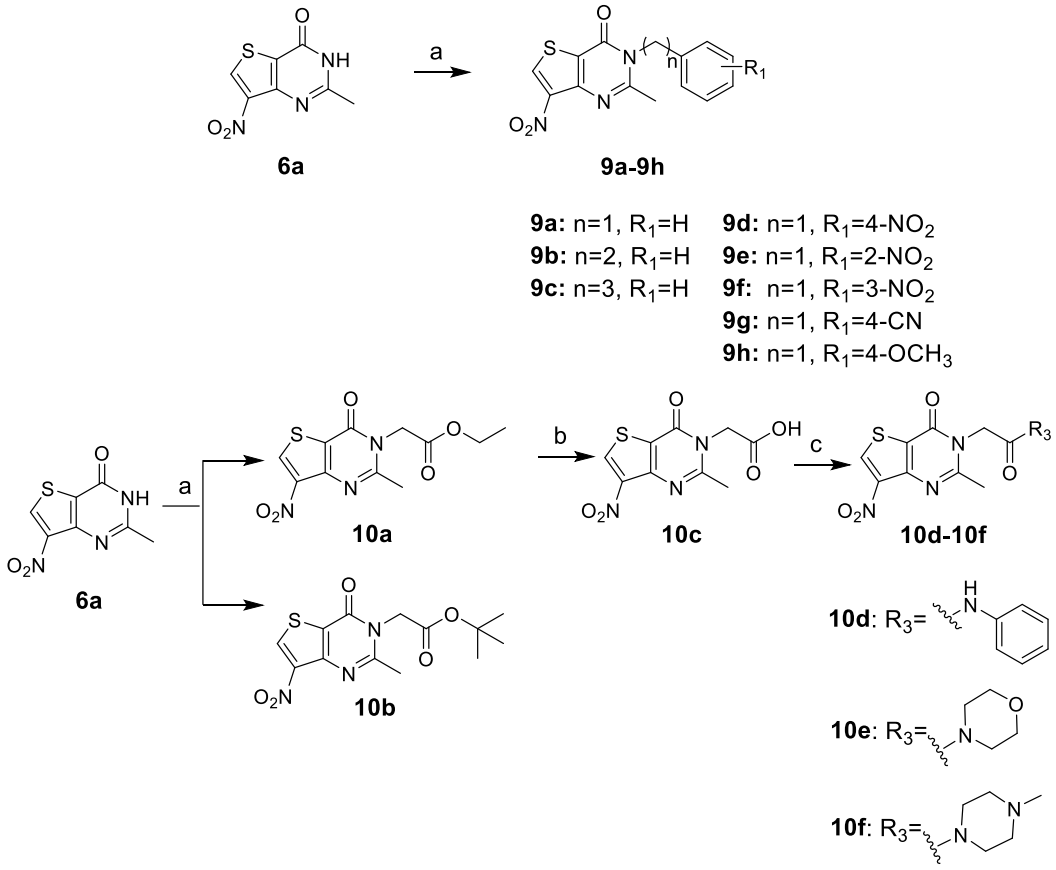
To pursue more potent compounds, lead **6a** was condensed with (un)substituted aromatic/heteroaromatic aldehydes using microwave conditions to obtain *C*2-styryl derivatives **8a**-**8n** (Scheme 3). [73,74]



Scheme 3. (a) benzaldehyde (for **8a**), furfural (for **8b**), 2-thiophenecarboxalehyde (for **8c**), 4-formylbenzoic acid (for **8d**), 5-formyl-2-furoic acid (for **8e**), 4-fluorobenzaldehyde (for **8f**), 2-fluorobenzaldehyde (for **8g**), 3-fluorobenzaldehyde (for **8h**), 4-nitrobenzaldehyde (for **8i**), 4-cyanobenzaldehyde (for **8j**), 4-chlorobenzaldehyde (for **8k**), 4-ethynylbenzaldehyde (for **8l**), 4-hydroxylbenzaldehyde (for **8m**), 4-methoxybenzaldehyde (for **8n**), AcOH, MW, 180 °C, 5-6 h, 20-37%.

4.1.4. Synthesis of *N*3-Substituted Derivatives

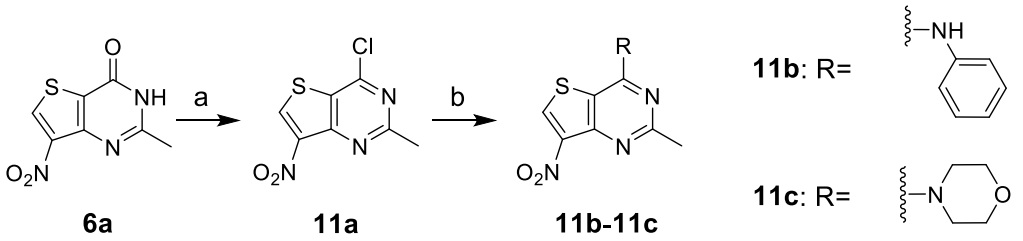
A wide range of substituents were installed at *N*3-position of **6a** as shown in Scheme 4. For example, **9a**-**9h** were prepared by reacting **6a** with various (un)substituted phenylalkyl halides in the presence of potassium carbonate in DMF.[39] To prepare analogues with *N*3-ester (**11a** and **11b**), -carboxylic acid (**11c**), and -amide (**11d**-**11f**) substituents, **6a** was subjected to alkylation using respective halides in the presence of potassium carbonate and DMF to obtain **11a** and **11b**.[39] The ester **11a** was hydrolyzed in the presence of lithium hydroxide to obtain **11c**, and the resulting carboxylic acid was coupled with aromatic/aliphatic amines to yield **11d**-**11f**.[52]



Scheme 4. (a) benzyl bromide (for **9a**), (2-bromoethyl)benzene (for **9b**), 1-bromo-3-phenylpropane (for **9c**), 4-nitrobenzyl bromide (for **9d**), 2-nitrobenzyl bromide (for **9e**), 3-nitrobenzyl bromide (for **9f**), 4-cyanobenzyl bromide, (for **9g**), 4-methoxybenzyl bromide (for **9h**), ethyl bromoacetate (for **10a**), *tert*-butyl 2-chloroacetate (for **10b**), K_2CO_3, DMF, rt, 8-12 h, 42-60%; (b) LiOH, THF, H_2O, rt, 8-10 h, 65-87%; (c) aniline (for **10d**), morpholine (for **10e**), *N*-methyl piperazine (for **10f**), EDC, HBTU, triethylamine, DCM, rt, 6h, 33-40%.

4.1.5. Synthesis of *C*4-Substituted Analogues

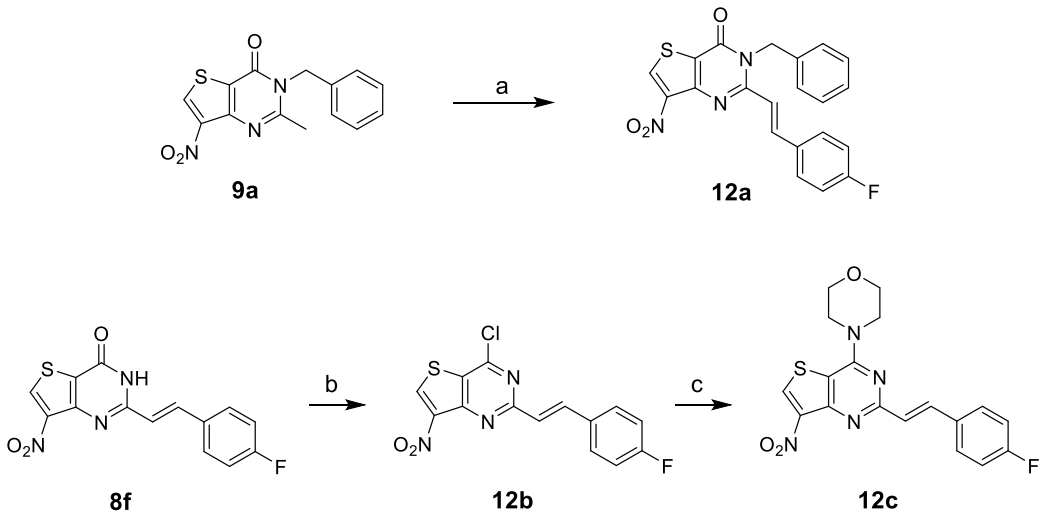
As depicted in Scheme 5, compound **11a** was prepared from the reaction of **6a** with phosphorus oxychloride. The *C*4-chloro group was then nucleophilically displaced by aniline or morpholine under microwave condition to obtain **11b** or **11c**.[53]



Scheme 5. (a) phosphorus oxychloride, reflux, 18 h, 40%, (b) aniline (for **11b**), morpholine (for **11c**), DMF, MW, 150 °C, 50-70 min, 35-45%.

4.1.6. Synthesis of Disubstituted Analogues of Thienopyrimidinone Core

Target compound **12a** with *C*2- and *N*3-disubstitution was prepared from the reaction of **9a** and 4-fluorobenzaldehyde. The 2,4-disubstituted compound **12b** was obtained by treating **8f** with phosphorus oxychloride, which upon nucleophilic substitution by morpholine led to **12c** as shown in Scheme 6.



Scheme 6. (a) 4-fluorobenzaldehyde, AcOH, MW, 180 °C, 4 h, 22%, (b) phosphorus oxychloride, reflux, 18 h, 72%; (c) morpholine, DMF, MW, 150 °C, 1 h, 32%.

4.2. Elucidation of Regioisomers from Nitration Reaction

Variety of techniques, including X-ray crystallography, 1-D (^{1}H and ^{13}C) and 2-D (HSQC and HMBC) NMR analyses, were employed to determine the regioselectivity of products obtained from the nitration reaction. We hypothesized that the position of the nitro group in representative compound **6a** would be at *C*7 instead of *C*6. This hypothesis was tested initially based on determination of the single X-ray crystal

structure of **6a**-derived analogue **10b**, and supported by ^{1}H and ^{13}C NMR chemical shifts analyses. Unambiguous assignment of regioisomer **11b** was accomplished by solving its single X-ray crystal structure (**Figure 5**).

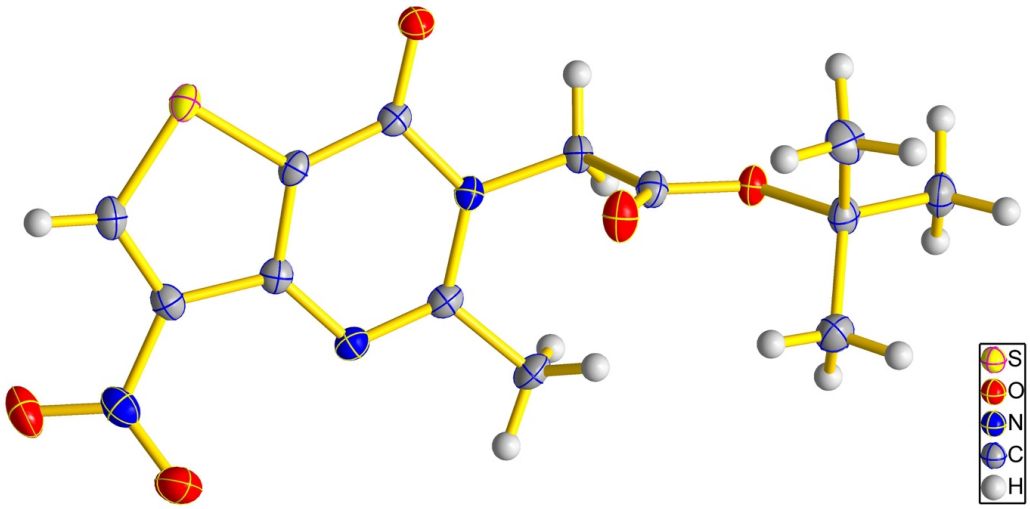


Figure 5. Crystal structure of compound **10b** (CCDC 1884213).

The *C*6-thiophene proton chemical shift of **6a** is 9.30 ppm and **10b** is 9.36 ppm (**Figure 6** and **Figure 7**), and the thiophene *C*6 chemical shift of **6a** and **10b** are 138.99 ppm and 139.98 ppm (**Figure 8** and **Figure 9**), respectively. These similar chemical shifts provide evidence for identical regioisomerism within compounds **6a** and **10b**. There is no distinct HMBC correlation present to differentiate the nitro group's position on the furan ring of compound **6b** (**Figure 10**), but reactivity of the starting material **7b** is similar to the sulfur-isostere analogue **7a**, from which compound **6a** was derived. Therefore, we believe that the nitro group is at 7-position in **6b** as well. HMBC spectra of **7b** is shown in supporting information (**Figure 11**).

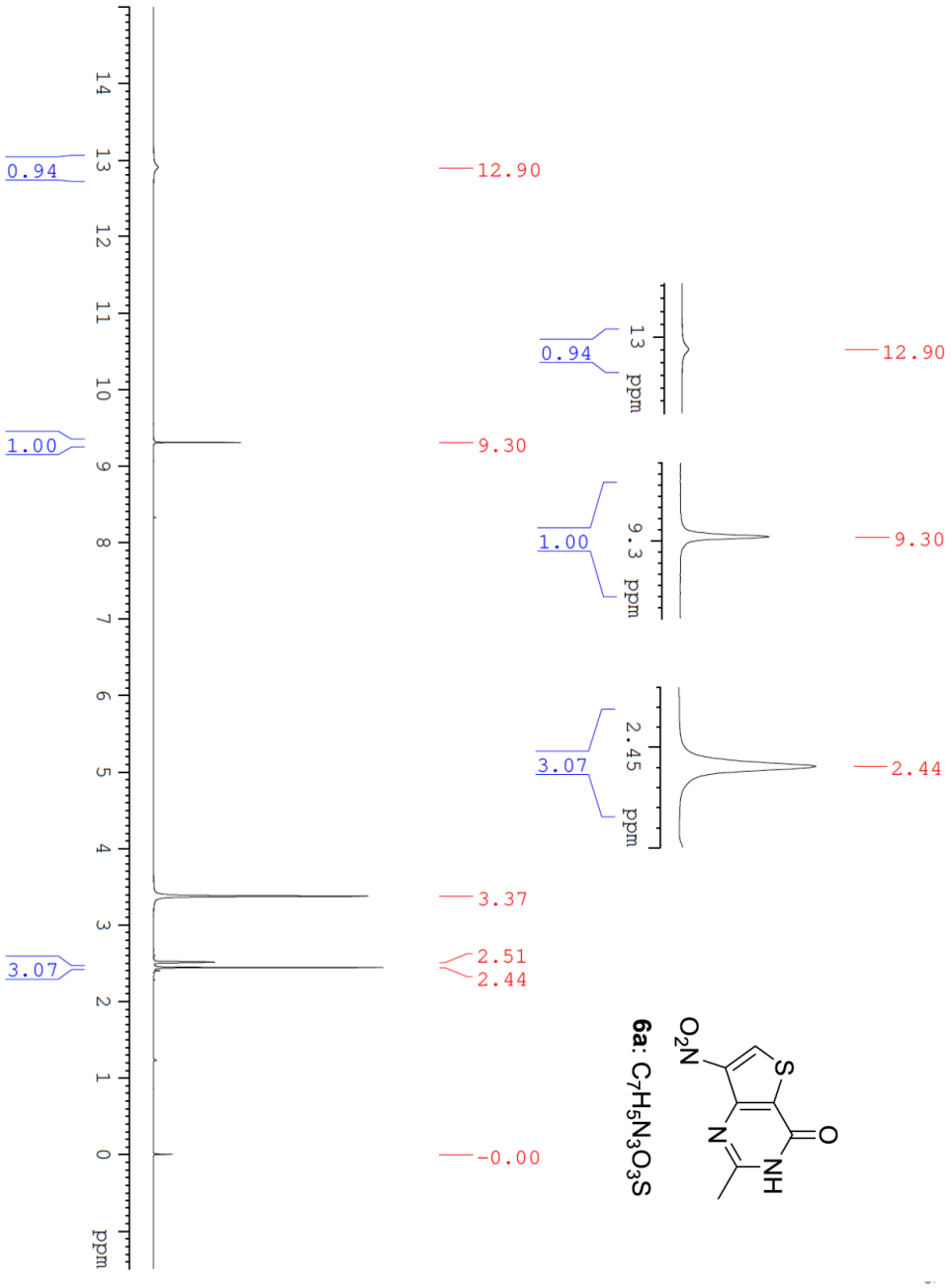


Figure 6. ^{1}H NMR spectra of **6a**.

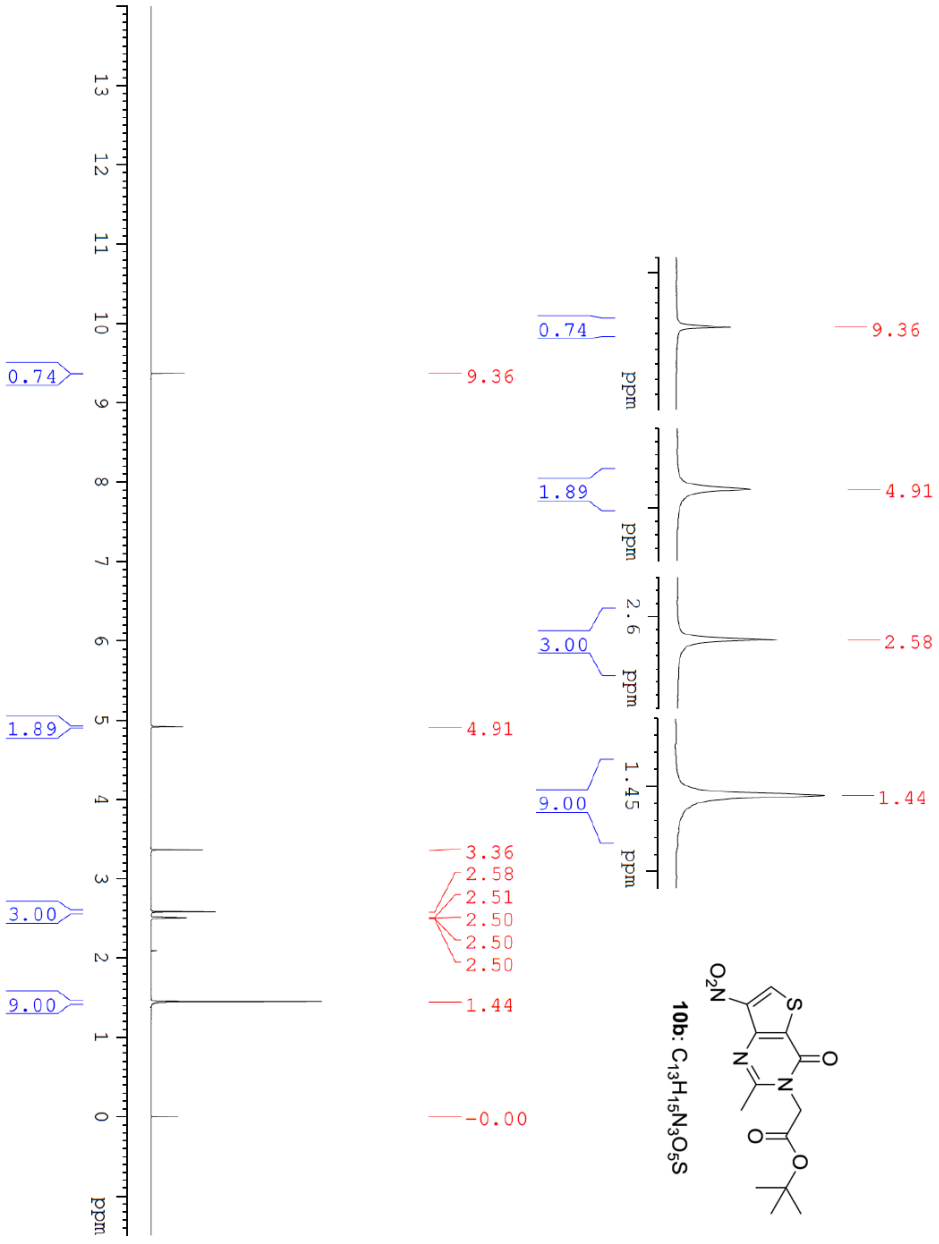


Figure 7. ^{1}H NMR spectra of **10b**.

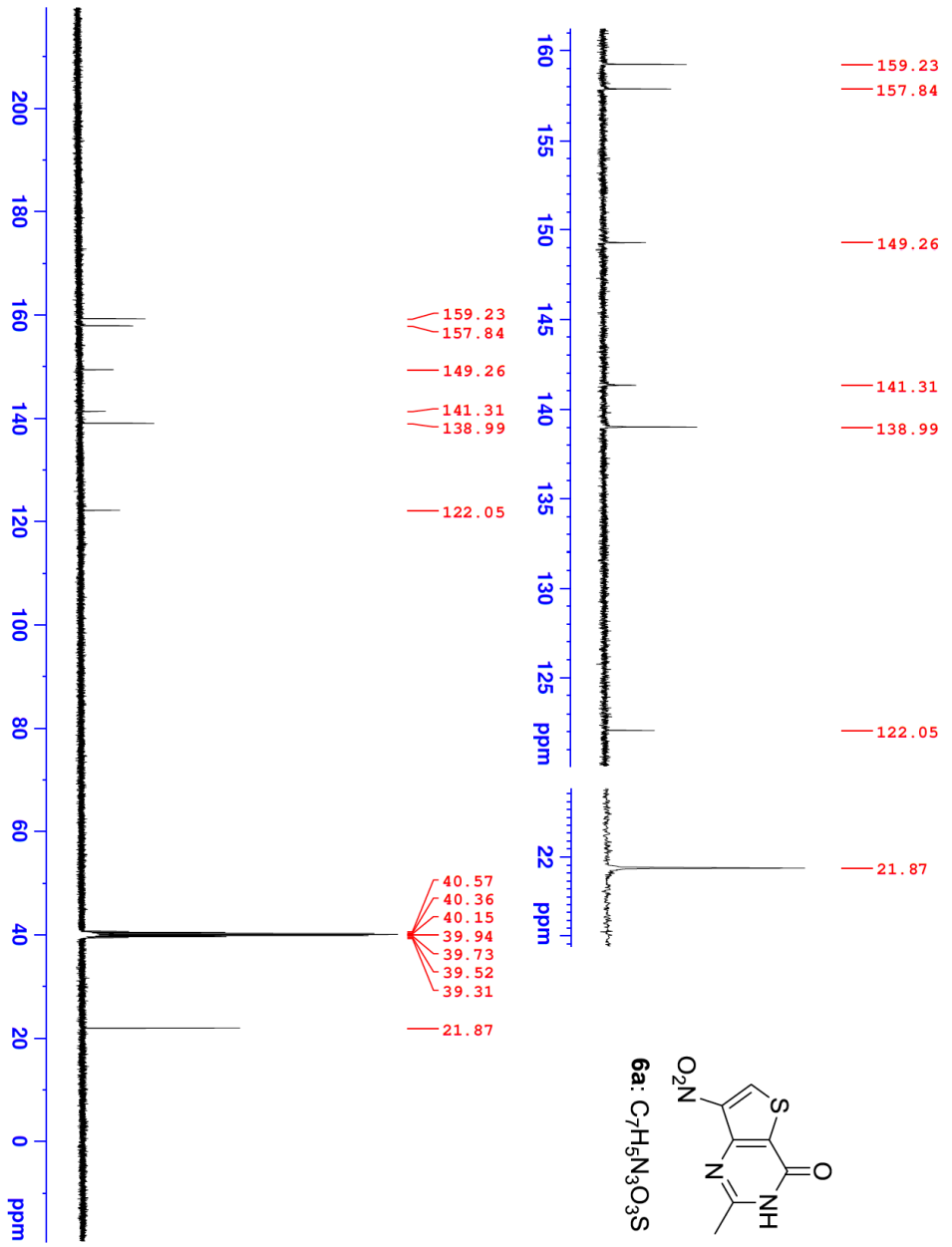


Figure 8. ^{13}C NMR spectra of **6a**.

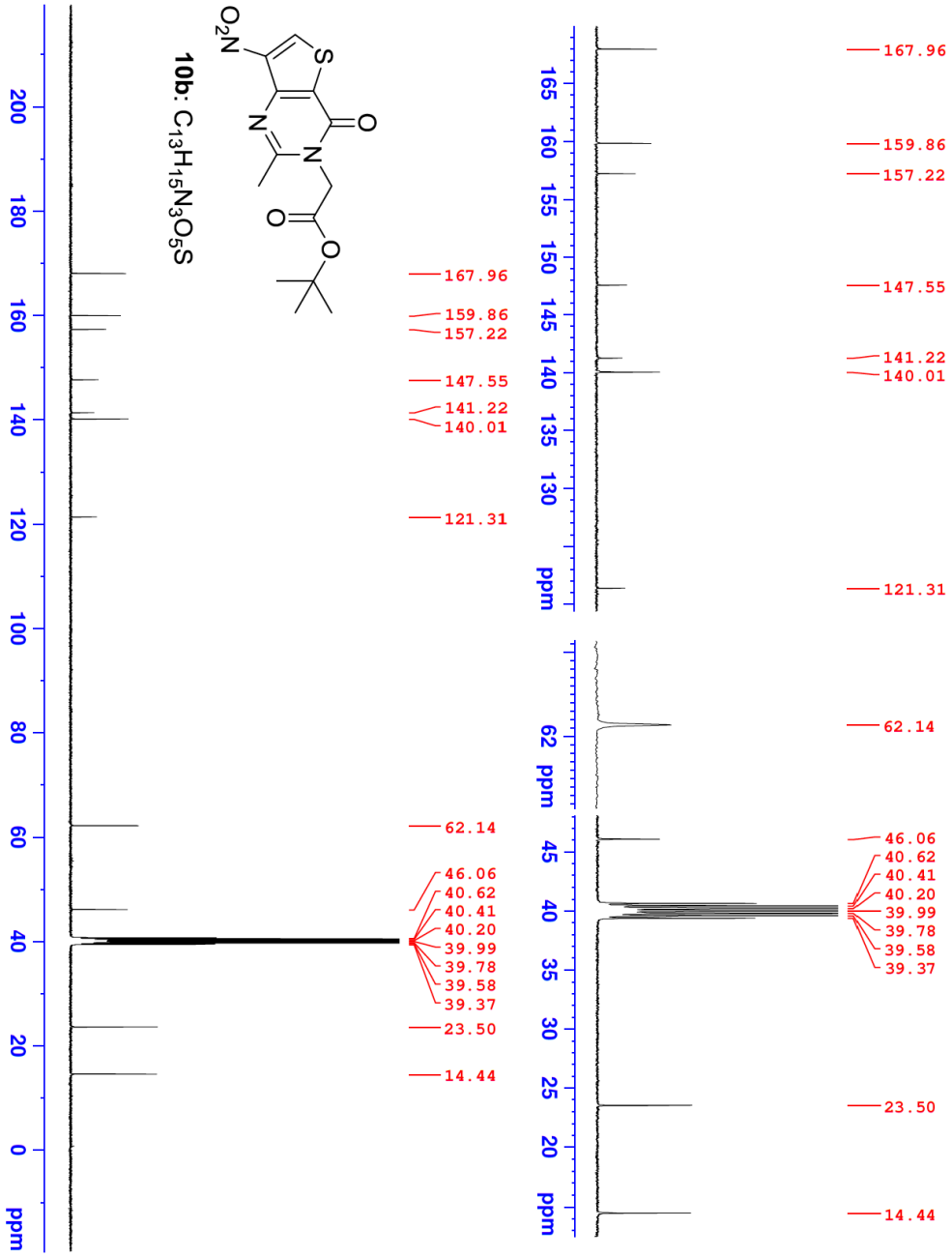


Figure 9. ^{13}C NMR spectra of **10b**.

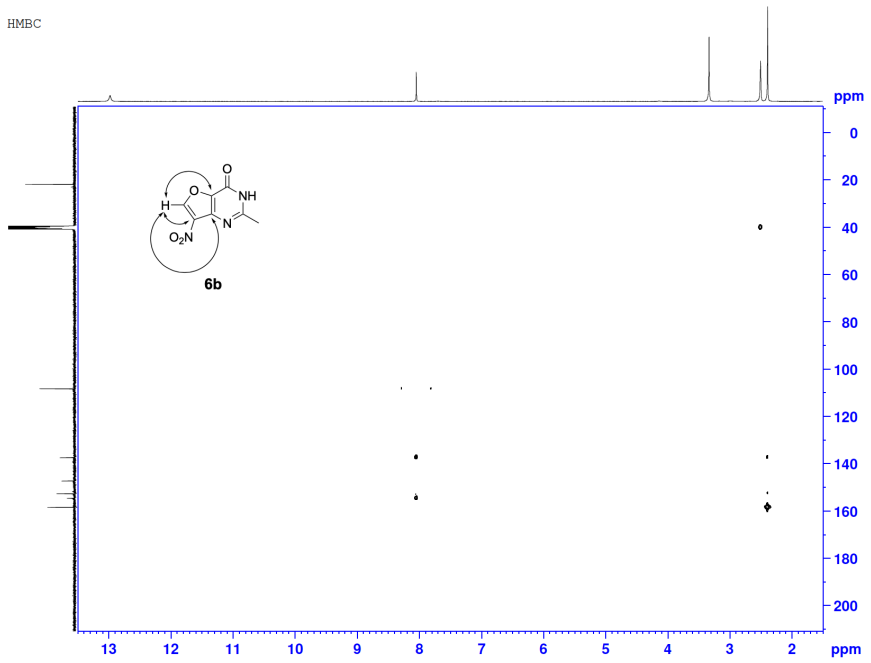


Figure 10. HMBC spectrum of **6b**.

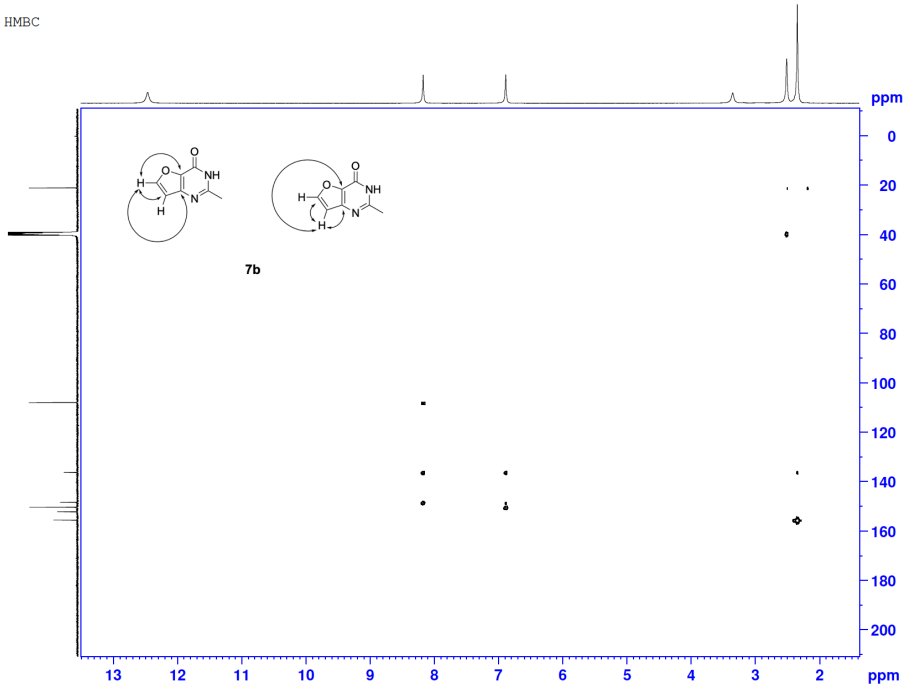


Figure 11. HMBC spectrum of **7b**.

Both HSQC and HMBC experiments were performed to investigate correlations between carbons and protons within compound **7c**, which was the starting material for

the nitration reaction to generate **6c**. The correlation between individual carbon atom and the proton attached to it was evidenced by the HSQC spectrum of **7c** (**Figure 12**). Additional HMBC analysis of **7c** shows four HMBC correlations for the hydrogen at 5-position, and three HMBC correlations for the hydrogen at 7-position as expected (**Figure 13**). Subsequent HMBC spectrum of compound **6c** shows four HMBC correlations for a single thiophene proton (**Figure 14**). These observations lead to the conclusion that the nitration reaction afforded the 7-nitro analogue as the exclusive regioisomer.

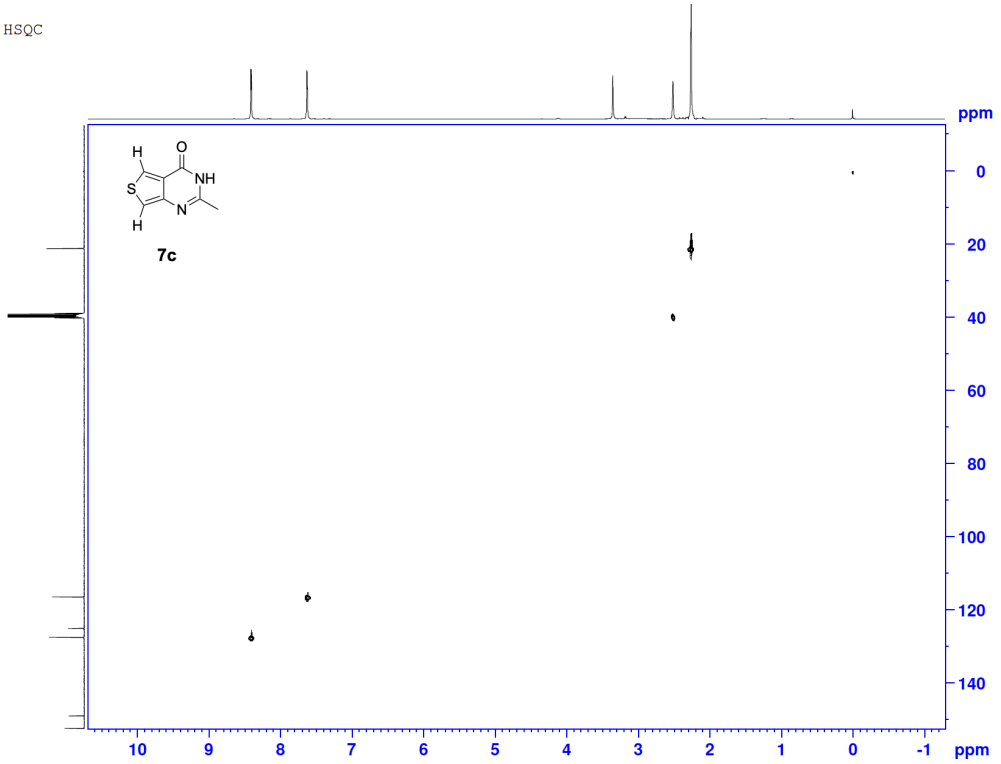


Figure 12. HSQC spectrum of **7c**.

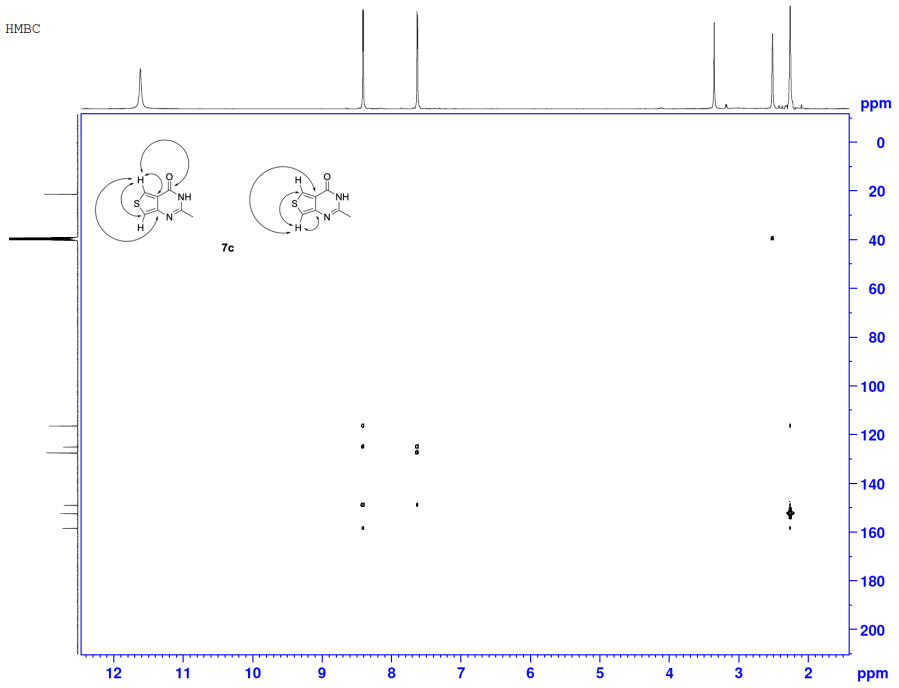


Figure 13. HMBC spectrum of **7c**.

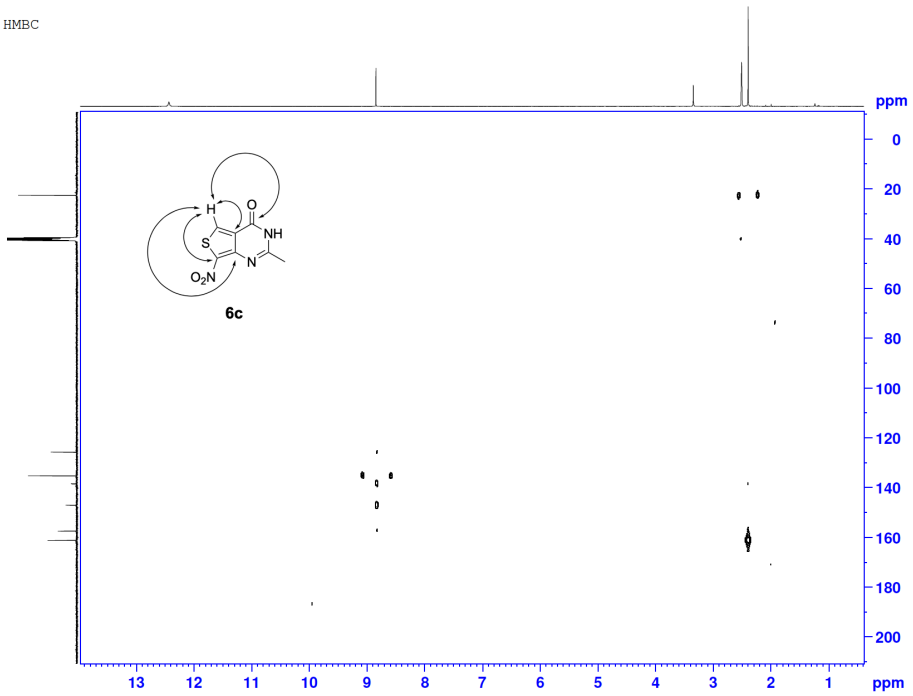
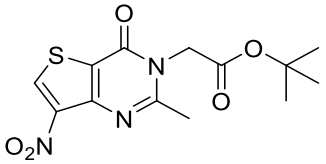
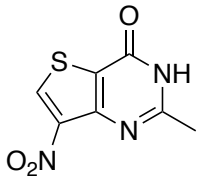
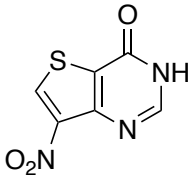
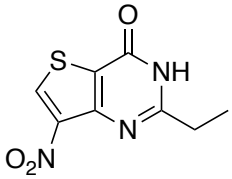
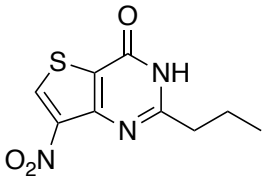
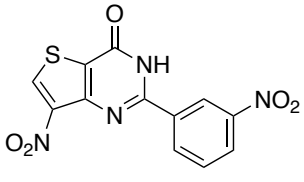


Figure 14. HMBC spectrum of **6c**.

For analogues **6d** – **6g**, which are structurally analogous to **6a**, we observed that the thiophene proton (9.31 – 9.41 ppm) and the carbon atom (138.89 – 139.21 ppm) with proton attached, have very similar chemical shift values compared to compound **6a** (^{1}H 9.30 ppm, ^{13}C 138.99 ppm) indicating the presence of nitro group at 7-position (**Table 3**).

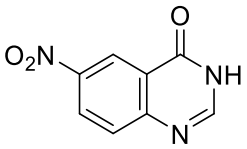
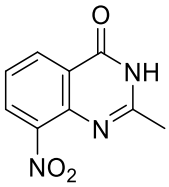
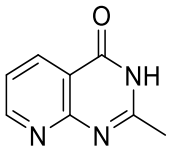
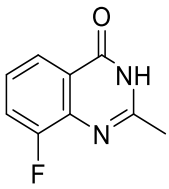
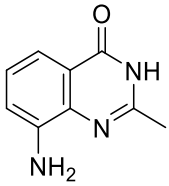
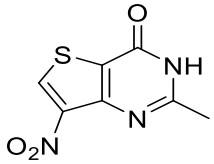
Table 3. ^{1}H and ^{13}C Chemical shift of compound **6a**, **6d** – **6g**, and **10b**

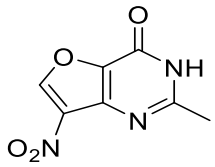
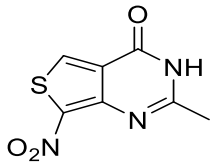
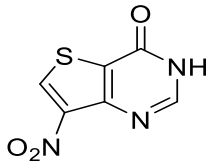
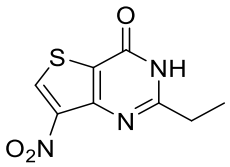
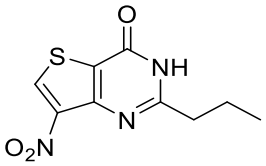
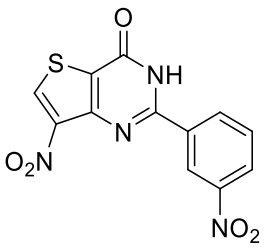
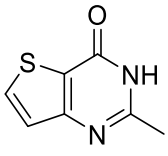
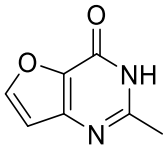
Compound	Structure	Chemical shift (ppm)	
		^{1}H	^{13}C
10b		9.36	139.98
6a		9.30	138.99
6d		9.36	139.21
6e		9.31	138.89
6f		9.31	138.92
6g		9.41	139.77

4.3. Structure-Activity Relationship

An in vitro antibacterial screening protocol was designed to identify compounds with potent inhibitory activity toward *C. difficile*, and minimal effect on normal human microflora and mammalian cells. To achieve this goal, we tested target compounds against two pathogenic strains of *C. difficile* (ATCC BAA 1870 and ATCC 43255) as shown in Tables 1-5. MIC values (μM and μg/mL) are used to describe antibacterial activities of the compounds and used for the interpretation of SAR data. An initial SAR study was focused on elucidation of the role of nitro substituent on hit-to-lead scaffolds **6a**-**6c** toward *C. difficile* inhibition as shown in Table 1. *Des*-nitro analogues of **6a**-**6c** gave **7a** (MIC >770/385 μM), **7b** (MIC >852/852 μM), and **7c** (MIC = 192/768 μM) with a substantial loss of anti-*C. difficile* potency. Other *des*-nitro derivatives such as **7d** (MIC = 420/840 μM), **7g** (MIC >560/>560 μM), **7h** (MIC >710/>710 μM), and **7i** (MIC >770/>770 μM) were also manifested with a loss of potency. These results allowed us to conclude the essential role of a nitro substituent at the *C*7-position. Next, we decided to elucidate the contribution of a *C*2-methyl group on anti-*C. difficile* potency (Table 3). Toward this goal, we prepared and tested *C*2-desmethyl (**6d**, MIC = 20/10 μM), *C*2-ethyl (**6e**, MIC = 35/35 μM), *C*2-propyl (**6f,** MIC = 67/67 μM) and *C*2-*meta*-nitrophenyl (**6g**, MIC = 50/50 μM) analogues exhibiting potency comparable to that of **6a**. Therefore, we hypothesized that further extensions at the *C*2-position may be tolerated while expanding chemical space to obtain potent compounds.

Table 4. MICof Compounds with Core Modifications

Compound	Structure	MIC μM (μg/mL)	
		ATCC BAA 1870	ATCC 43255
1		335 (64)	335 (64)
2		312 (64)	156 (32)
3		>794 (>128)	>794 (>128)
4		>718 (>128)	>718 (>128)
5		>730 (>128)	>730 (>128)
6a		19 (4)	38 (8)

6b		41 (8)	41 (8)
6c		38 (8)	38 (8)
6d		20 (4)	10 (2)
6e		35 (8)	35 (8)
6f		67 (16)	67 (16)
6g		50 (16)	50 (16)
7a		>770 (>128)	385 (64)
7b		>852 (>128)	>852 (>128)

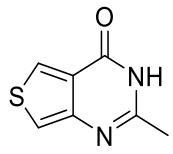
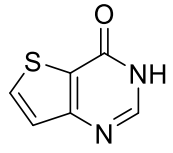
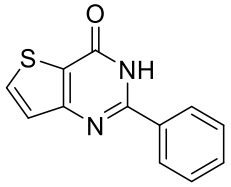
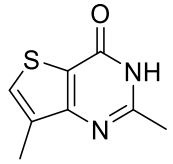
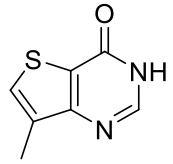
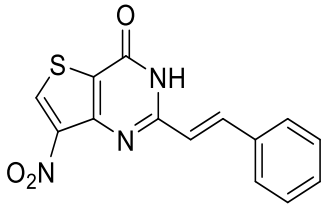
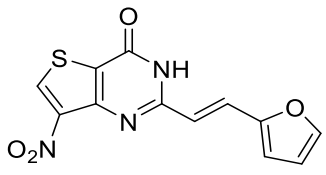
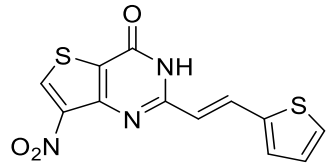
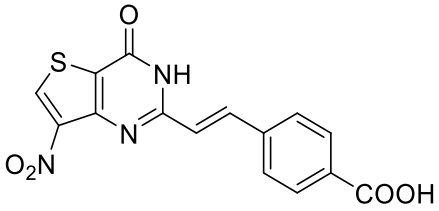
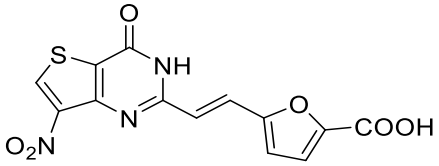
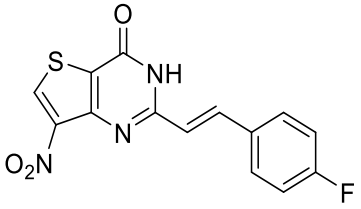
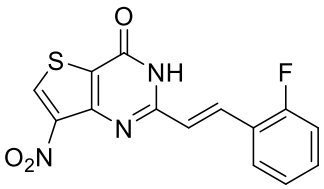
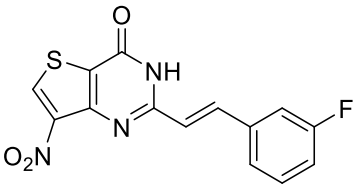
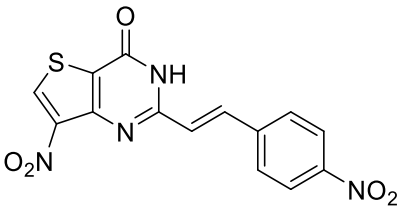
7c		192 (32)	768 (128)
7d		420 (64)	840 (128)
7g		>560 (>128)	>560 (>128)
7h		>710 (>128)	>710 (>128)
7i		>770 (>128)	>770 (>128)
Vancomycin	-	0.7 (1)	0.3 (0.5)
Metronidazole	-	0.7 (0.125)	1.5 (0.25)
Fidaxomicin	-	0.1 (0.0625)	0.1 (0.0625)

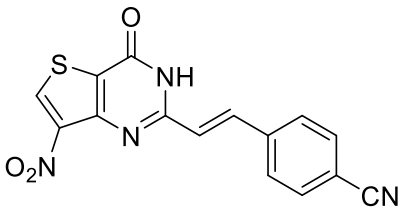
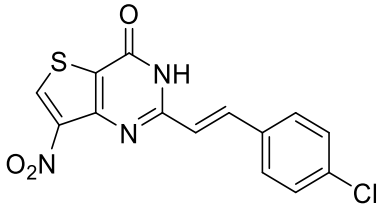
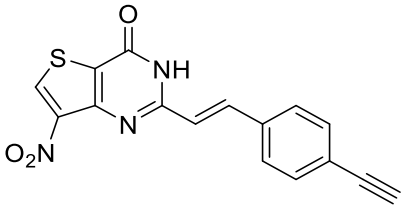
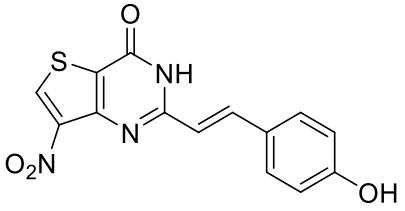
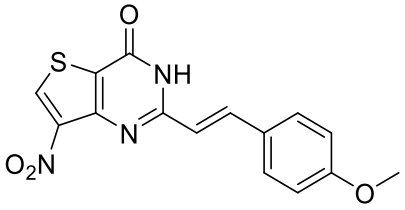
Table 5 shows the MICs of *C*2-styryl analogues. The *C*2-styryl derivative **8a** yielded a promising MIC of 13/52 μM. Considering the favorable result, we replaced the phenyl ring with isosteres such as furan-2-yl (**8b**, MIC = 27/27 μM) and thiophen-2-yl (**8c**, MIC = 13/13 μM) which suggested the tolerance for these isosteric replacements. Next, we prepared carboxy substituted derivatives of **8a** and **8b** leading to **8d** (MIC = 46/184 μM) and **8e** (MIC >384/>384 μM) with a considerable loss of potency. This loss of potency may be attributed to unfavorable interactions with the

target and/or poor permeability. On the contrary, 4-fluorophenyl analogue (**8f**, MIC = 3/6 μM) showed excellent potency. A fluoro-scan was conducted to identify the most favorable position for a fluoro group, which led to 2-fluoro (**8g**, MIC = 6/12 μM) and 3-fluoro (**8h**, MIC = 13/13 μM) analogues. Since 4-fluoro analogue yielded the potent MIC value, we explored substitutions of different electron-withdrawing/-donating groups at the 4-position. While 4-nitro analogue (**8i**, MIC = 24/12 μM) gave comparable activity, the 4-cyano analogue (**8j**, MIC >197/>197 μM) proved detrimental. The 4-chloro analogue (**8k**, MIC = 6/6 μM) being a classical isostere of 4-fluoro group retained potency similar to that observed for the 4-fluoro analogue. Retention of potency by 4-acetylene derivative (**8l**, MIC = 6/12 μM) indicated a tolerance for the conformationally rigid acetylene group. Similar to the electron-withdrawing groups, electron-donating groups also showed favorable potency as exemplified by 4-hydroxy (**8m**, MIC = 26/13 μM) and 4-methoxy (**8n**, MIC = 12/12 μM) analogues.

Table 5. MIC of Compounds with *C*2 Substitutions

Compound	Structure	MIC μM (μg/mL)	
		ATCC BAA 1870	ATCC 43255
8a		13 (4)	52 (16)
8b		27 (8)	27 (8)

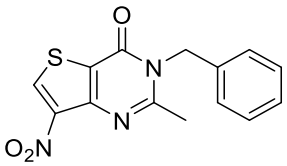
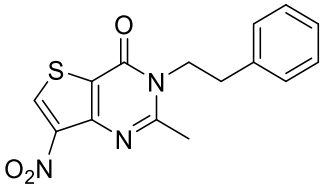
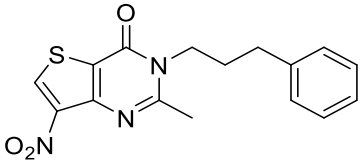
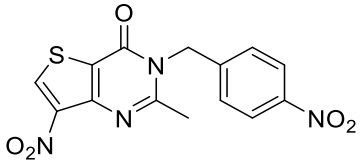
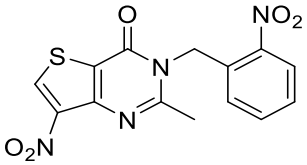
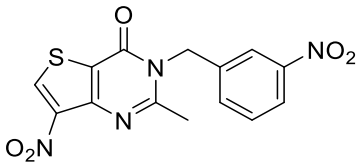
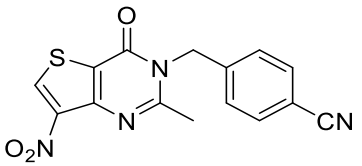
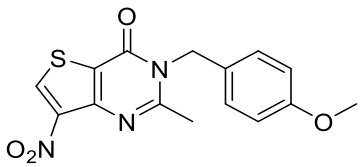
8c		13 (4)	13 (4)
8d		46 (16)	184 (64)
8e		>384 (>128)	>384 (>128)
8f		3 (1)	6 (2)
8g		6 (2)	12 (4)
8h		13 (4)	13 (4)
8i		24 (8)	12 (4)

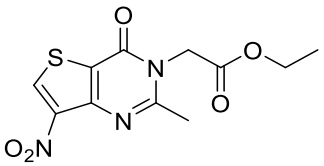
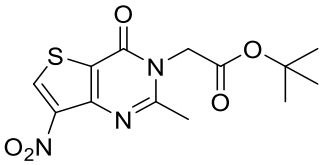
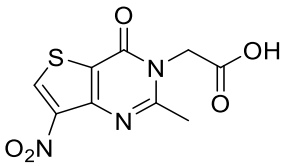
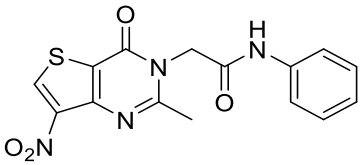
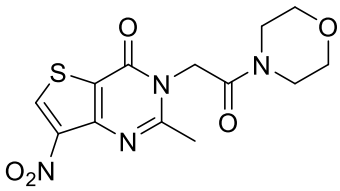
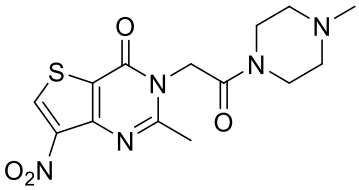
8j		>197 (>64)	>197 (>64)
8k		6 (2)	6 (2)
8l		6 (2)	12 (4)
8m		26 (8)	13 (4)
8n		12 (4)	12 (4)
Vancomycin	-	0.7 (1)	0.3 (0.5)
Metronidazole	-	0.7 (0.125)	1.5 (0.25)
Fidaxomicin	-	0.1 (0.0625)	0.1 (0.0625)

The next SAR included investigation of various *N*3-substituents in lead **6a** as shown in Table 6. At the onset, we inserted *N*3-substituents such as benzyl (**9a**, MIC = 7/56 μM), phenylethyl (**9b**, MIC = 13/13 μM) and phenylpropyl (**9c**, MIC = 12/12 μM)

to investigate the influence of varying linker length and obtained comparable potency to that observed for **6a**. Based on these analogues, it may be suggested that the binding pocket of *N*3-substituents is located further from the *N*3-position. Based on the favorable MIC values and straightforward derivatization, we chose benzyl analogue **9a** for further SAR study. Scanning of the nitro group on the benzyl moiety at different positions produced 4-nitro (**9d**, MIC = 6/12 μM), 2-nitro (**9e**, MIC = 23/23 μM) and 3-nitro (**9f**, MIC = 23/23 μM) analogues. Considering favorable contribution of 4-substituents, we prepared 4-cyano analogue (**9g**, MIC = 25/50 μM), which showed 4-fold reduced potency. The electron-donating 4-methoxy analogue (**9h**, MIC = 24/24 μM) also suffered a loss of activity compared to **9d**. These findings prompted us to investigate untapped chemical space around **6a**, which led to *N*3-ethoxycarbonyl methylene analogue (**10a**, MIC = 54/54 μM), *N*3-*tert*-butyloxycarbonyl methylene analogue (**10b**, MIC = 49/98 μM), and methylene carboxylic acid (**10c**, MIC >475/>475 μM). This data suggested the tolerance for an ester group at *N*3-position. The detrimental effect of a carboxyl group in **10c** may be due to poor permeability through *C. difficile* cell membrane and/or unfavorable interaction with the target, which is consistent with the activity loss observed for the other carboxylic acid analogues **8d**, and **8e**. Thus, we decided to convert unfavorable carboxyl group to an amide by coupling it with an aniline, morpholine, and *N*-methylpiperazine, respectively, to produce **10d** (MIC = 46/23 μM), **10e** (MIC = 47/94 μM), and **11f** (MIC = 91/91 μM) with a >5-fold recovery of activity compared to the carboxyl analogue **10c**.

Table 6. MIC of Compounds with *N*3 Substitutions

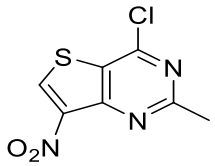
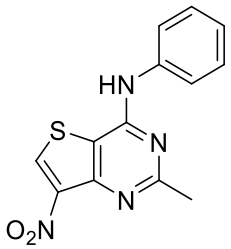
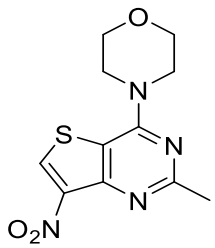
Compound	Structure	MIC μM (μg/mL)	
		ATCC BAA 1870	ATCC 43255
9a		7 (2)	56 (16)
9b		13 (4)	13 (4)
9c		12 (4)	12 (4)
9d		6 (2)	12 (4)
9e		23 (8)	23 (8)
9f		23 (8)	23 (8)
9g		25 (8)	50 (16)
9h		24 (8)	24 (8)

10a		54 (16)	54 (16)
10b		49 (16)	98 (32)
10c		>475 (>128)	>475 (>128)
10d		46 (16)	23 (8)
10e		47 (16)	94 (32)
10f		91 (32)	91 (32)
Vancomycin	-	0.7 (1)	0.3 (0.5)
Metronidazole	-	0.7 (0.125)	1. 5 (0.25)
Fidaxomicin	-	0.1 (0.0625)	0.1 (0.0625)

Our next SAR involved expansion of a chemical space around *C*4-position of **6a**, which led to synthesis of *C*4-chloro derivative (**11a**, MIC = 17/34 μM) as shown in Table 7. We noticed that **11a** featured a significant change in the scaffold structure (transforming sp^3-hybridized *N*3 to sp^2-hybridized *N*3) with increased planarity, and still exhibited comparable activity to that of **6a**. We took advantage of the ease of nucleophilically displacing the *C*4-chloro with aniline and morpholine to obtain **11b**

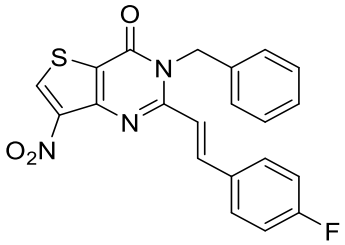
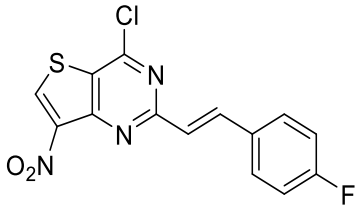
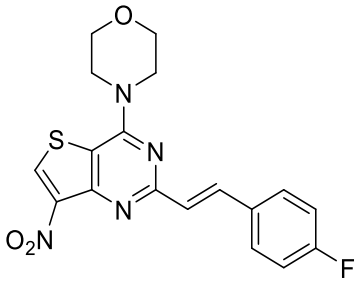
(MIC = 28/14 μM) and (**11c**, MIC = 14/14 μM) with essentially retention of activity as that of **11a**.

Table 7. MIC of Compounds with *C*4 Substitutions

Compound	Structure	MIC μM (μg/mL)	
		ATCC BAA 1870	ATCC 43255
11a		17 (4)	34 (8)
11b		28 (8)	14 (4)
11c		14 (4)	14 (4)
Vancomycin	-	0.7 (1)	0.3 (0.5)
Metronidazole	-	0.7 (0.125)	1.5 (0.25)
Fidaxomicin	-	0.1 (0.0625)	0.1 (0.0625)

The SAR data gathered so far indicated the favorable contribution of *C*2-arylidene, *N*3-benzyl and *C*4-chloro derivatives for *C. difficile* growth inhibition. Next, we decided to combine these structural features into a single molecule to evaluate whether additive effect on potency could be achieved (Table 8).

Table 8. MIC of Compounds with *C*2-and *N*3- or *C*2- and *C*4-Disubstitutions

Compound	Structure	MIC μM (μg/mL)	
		ATCC BAA 1870	ATCC 43255
12a		157 (64)	157 (64)
12b		95 (32)	95 (32)
12c		166 (64)	166 (64)
Vancomycin	-	0.7 (1)	0.3 (0.5)
Metronidazole	-	0.7 (0.125)	1. 5 (0.25)
Fidaxomicin	-	0.1 (0.0625)	0.1 (0.0625)

This has led to the synthesis and testing of *C*2-, *N*3-disusbtituted analogue (**12a**, MIC = 157/157 μM) with substantial loss of activity. The *C*2-, *C*4-disubstituted analogues **12b** (MIC = 95/95 μM) and **12c** (MIC = 166/166 μM) were also proved detrimental. This data suggests that the disubstitutions oppose each other's productive binding within respective pockets of the target. Overall summary of the SAR findings is depicted in Figure 15.

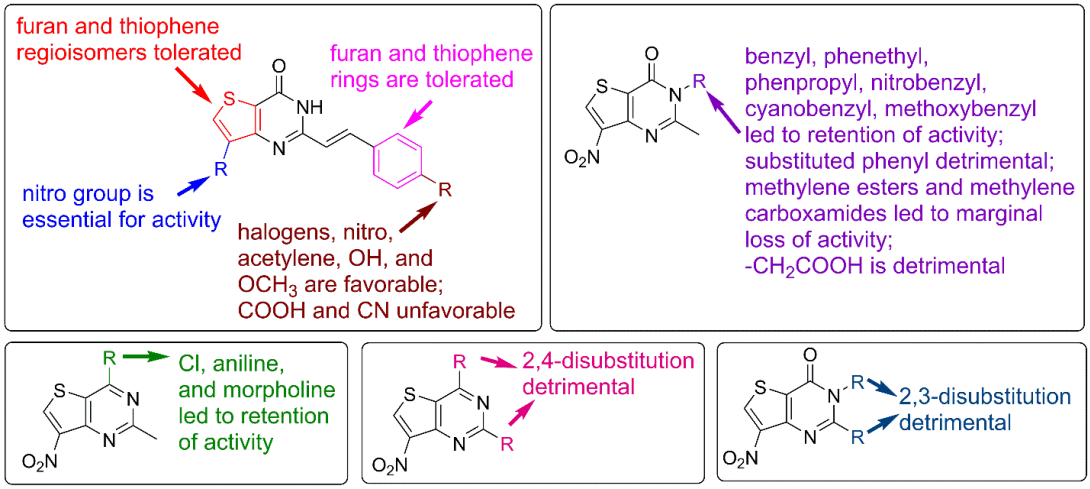


Figure 15. SAR summary of the thienopyrimidinone series of anti-*C. difficille* agents.

4.4. Killing kinetics of 8f against *C. difficile*

After exploration of the chemical space around lead compound **6a**, we sought to investigate the killing kinetics of the most potent compound in the series, **8f**. The minimum bactericidal concentration (MBC) of compound **8f** was 6 μM, which is two-fold higher than its MIC. We concluded that compound **8f** is bactericidal, based on the fact that the MBC value is lower than 3 X MIC value. To confirm the bactericidal activity of compound **8f**, we performed a time-kill assay and compared killing kinetics of **8f** to the standard anti-clostridial drugs, vancomycin and fidaxomicin, at 8 X MIC. Interestingly, compound **8f** completely eradicated the bacteria in 6 h after incubation as opposed to 24 h required for eradication by fidaxomicin (**Figure 16**).

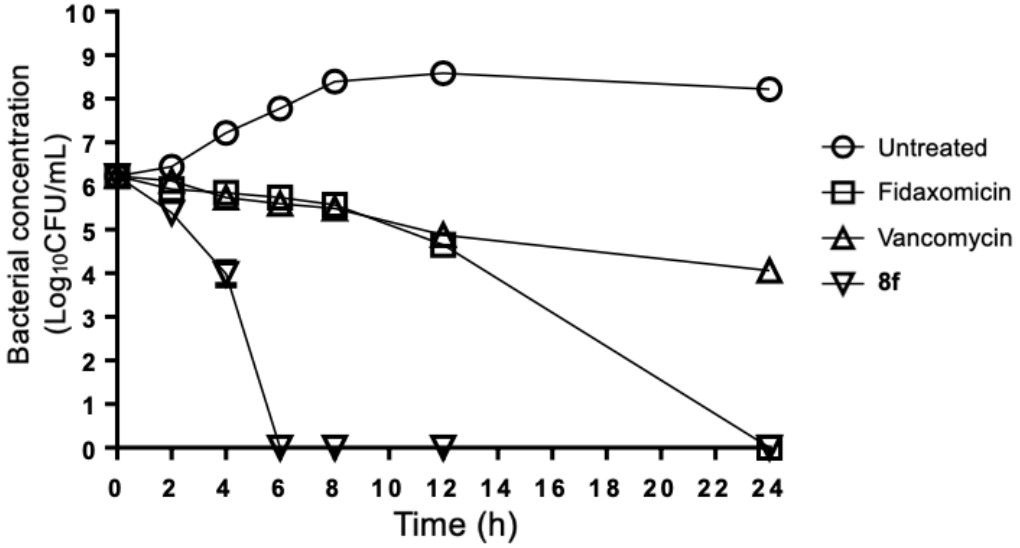


Figure 16. Time-kill assay of compound **8f** against *C. difficile* ATCC BAA 1870.

4.5. Selectivity of representative compounds against *C. difficile* over normal cells

We next investigated the toxicity of representative compounds (**6a**, **8f**, **8g**, **8k**, **9a**, **9c**, **9d** and **11c**) against human and animal cells. As presented in Figure 17, the cytotoxicity of the most potent compounds was tested against three cell lines; human colon colorectal adenocarcinoma (Caco-2 cells), human ileocecal adenocarcinoma (HRT-18 cells) and African green monkey kidney cells (Vero cells). All tested compounds showed no toxicity against the tested cell lines at >256 μM.

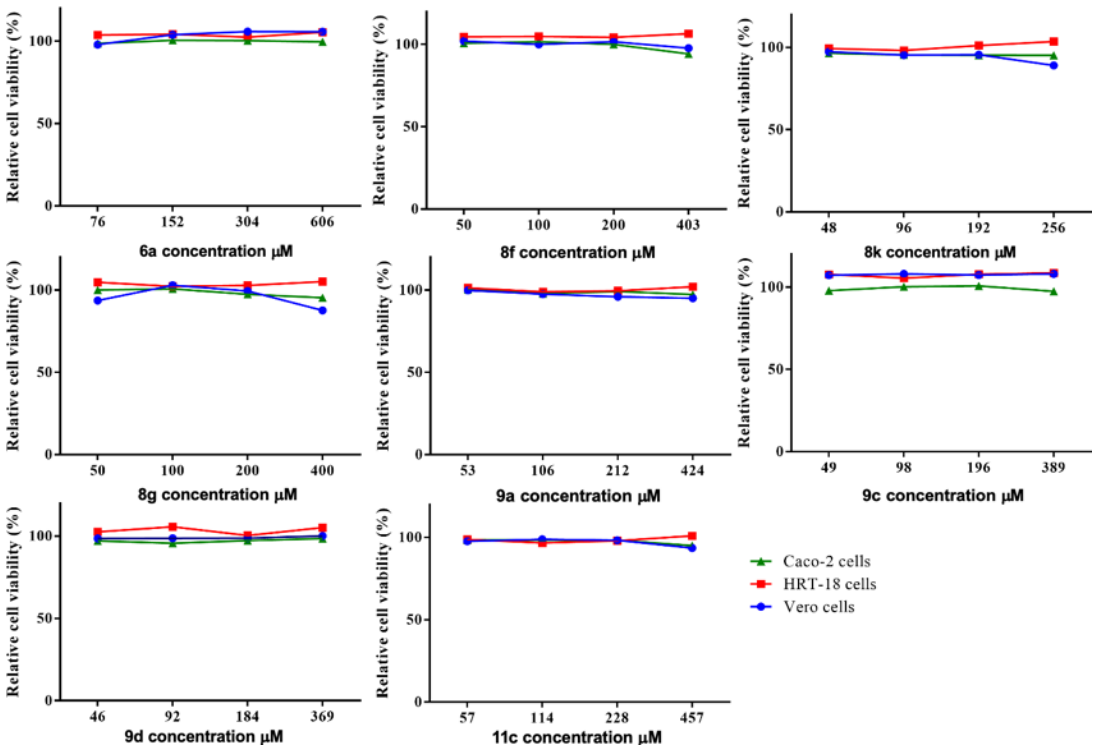


Figure 17. In vitro cytotoxicity evaluation of the most potent compounds against 3 different cell lines; human colon colorectal adenocarcinoma (Caco-2), human ileocecal adenocarcinoma (HRT-18) and African green monkey kidney cells (Vero).

4.6. Selectivity of representative compounds against *C. difficile* over gut flora

Since the killing of beneficial gut microflora leads to a significant growth of opportunistic pathogens such as *C. difficile* and subsequent colonization and recurrence of CDI, it is important to determine whether representative set of compounds (**6a**, **8f**, **8g**, **8k**, **9a**, **9c**, **9d** and **11c**) are selective toward *C. difficile* while sparing normal gut microflora. These compounds did not show any activity against human normal gut bacteria at >425 μM, including *Lactobacillus*, *Bifidobacterium*, *E. coli*, and *Enterobacter cloacae* (Table 9). On the contrary MICs of positive controls (vancomycin, fidaxomicin and metronidazole) ranged from 1 – 1496 μM.

Table 9. Activity of Selected Compounds against Human Normal Flora[a]

	MIC (μM)											
	6a	**8f**	**8g**	**8k**	**9a**	**9c**	**9d**	**11c**	**Vancomycin**	**Metronidazole**	**Fidaxomicin**	**Gentamicin**
***Lactobacillus gasseri* HM-400**	1212	>807	>807	>767	>425	>777	>739	>913	≤1	>1496	≤2	NT
***Lactobacillus casei* ATCC 334**	>1212	>807	>807	>767	>425	>777	>739	>913	>177	47	>242	NT
***Lactobacillus crispatus* HM-103**	>1212	>807	>807	>767	>425	>777	>739	>913	≤1	>1496	30	NT
***Bifidobacterium bifidum* ATCC 11863**	>1212	>807	>807	>767	>425	>777	>739	>913	>177	>1496	>242	NT
***Escherichia coli* ATCC 25922**	>1212	>807	>807	>767	>850	>777	>739	>913	NT	NT	NT	8
***Enterobacter cloacae* ATCC BAA-1143**	>1212	>807	>807	>767	>850	>777	>739	>913	NT	NT	NT	≤1

NT = Not tested

4.7. Aqueous solubility and SGF/SIF stability of representative compounds

Aqueous solubility plays an important role in determining the suitability of thienopyrimidinone derivatives for targeting the site of action i.e., intestine and also to facilitate preclinical to clinical transition, therefore we determined the solubility of representative potent compounds in PBS buffer (pH 7.3 to 7.5). Compound **6a** showed >500 μg/mL solubility in PBS buffer whereas compounds **8f**, **9c**, **9d**, and **11c** exhibited lower solubility ranging from 10 to 20 μg/mL. Stability of these compounds in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) was also evaluated at two time points (Table 10). We observed that SGF stability followed the order of **9d>6a>8f>9c>11c**, whereas SIF stability was in the order of **9d>6a>11c>8f>9c**.

Table 10. Aqueous Solubility in PBS Buffer (pH 7.3 – 7.5) and Stability in SGF (Simulated Gastric Fluid) / SIF (Simulated Intestinal Fluid) of Representative Thienopyrimidinone Analogues

Compound	Aqueous solubility[a] (μg/mL)	Percentage remaining in SGF 4 h	Percentage remaining in SGF 8 h	Percentage remaining in SIF 4 h	Percentage remaining in SIF 8 h
6a	>500	90%	90%	91%	76%
8f	14.79	50%	48%	60%	51%
9c	10.76	45%	<10%	<10%	<10%
9d	12.73	>95%	>95%	>95%	90%
11c	18.69	<10%	<10%	79%	80%

[a] Aqueous solubility was determined in PBS buffer at pH 7.3 – 7.5.
metronidazole: 10 mg/mL in water;[75]
vancomycin hydrochloride: 17.6 mg/mL in water;[76]
fidaxomicin: 18 μg/mL at pH 7.

4.8. In Silico PAINS Analysis.

All the synthesized target compounds were subjected to PAINS filters by using a KNIME (v3.74, KNIME GmbH, Konstanz, Germany) workflow.[70] Molecular formula strings of target compounds were manually input into the workflow, and the output file for the run indicated no PAINS were found.

Conclusions

The SAR data gathered during hit identification and hit-to-lead exploratory medicinal chemistry revealed key pharmacophore features of thienopyrimidinone series that contributed to the potency against clinical strains of *C. difficile*. These observations included: a) the nitrophenyl portion of the screening hit **2** can be replaced with isosteric nitrothienyl and nitrofuranyl to enhance potency in ligand-efficient fashion (e.g., **2** vs **6a**-**6c**), b) the *C*2-methyl substitution does not significantly influence potency (e.g., **6a** vs **6d**-**6f)**, c) the presence of an electron-withdrawing regiospecific nitro group on a bicyclic scaffold is indispensable to the potency of these compounds (e.g., **1** vs **2**, **7a** vs **6a**, **7b** vs **6b**, and **7c** vs **6c**), d) the *C*2-methyl can be extended to an aryl/heteroaryl ethenyl moiety without significant loss of potency (e.g., **6a** vs **8a-8c**, **8f-8i** and **8k**-**8m**), e) the 3-NH of thienopyrimidinone scaffold can be substituted with various arylalkyl moieties, aliphatic esters or aliphatic amides (e.g., **6a** vs **9a**, **9d**-**9h**, **10a**, **10b** and **10d**-**10f**) with retention of potency, f) the *C*4 carbonyl oxygen can be replaced with chloro and aromatic/aliphatic amines with retention of activity (e.g., **6a** vs **11a**-**11c**), g) either *C*2-, *N*3- or *C*2-, *C*4-disubstitutions on pyrimidine ring of **6a** proved detrimental (e.g., **6a** vs **12a**-**12c**), and h) compounds (e.g., **8d**, **8e**, and **11c**) with the carboxylic acid substituent showed detrimental activities. The most promising compound (**8f**) from this series exhibited excellent profile: i) potent and rapid killing against *C. difficile* strains, ii) excellent selectivity over human normal flora, iii) low cytotoxicity against mammalian cells, iv) increased GI stability, and v) desirable aqueous solubility. Unlike synthetically intractable and architecturally complex macrocyclic antibiotics, vancomycin (MW = 1449 Da) and fidaxomicin (MW = 1058 Da) that are difficult to structurally optimize, the current series of small MW thienopyrimidinones offer

significant scope for further medicinal chemistry optimization to explore SAR, and improve in vitro activity without increasing the molecular size and complexity beyond 600 Da.

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