Bioorganic & Medicinal Chemistry 28 (2020) 115439

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Identification of an ethyl 5,6-dihydropyrazolo[1,5-*c*]quinazoline-1-carboxylate as a catalytic inhibitor of DNA gyrase

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ARTICLE INFO

Keywords:
DNA gyrase
Quinazolinediones
Fluoroquinolones
Topoisomerase

ABSTRACT

Fluoroquinolones are a class of antibacterial agents used clinically to treat a wide array of bacterial infections and target bacterial type-II topoisomerases (DNA gyrase and topoisomerase IV). Fluoroquinolones, however potent, are susceptible to bacterial resistance with prolonged use, which limits their use in the clinic. Quinazoline-2,4-diones also target bacterial type-II topoisomerases and are not susceptible to bacterial resistance similar to fluoroquinolones, however, their potency pales in comparison to fluoroquinolones. To meet the increasing demand for antibacterial development, nine modified quinazoline-2,4-diones were developed to probe quinazoline-2,4-dione structure modification for possible new binding contacts with the bacterial type-II topoisomerase, DNA gyrase. Evaluation of compounds for inhibition of the supercoiling activity of DNA gyrase revealed a novel ethyl 5,6-dihydropyrazolo[1,5-*c*]quinazoline-1-carboxylate derivative as a modest inhibitor of DNA gyrase, having an IC_{50} of 3.5 μM. However, this ethyl 5,6-dihydropyrazolo[1,5-*c*]quinazoline-1-carboxylate does not trap the catalytic intermediate like fluoroquinolones or typical quinazoline-2,4-diones do. Thus, the ethyl 5,6-dihydropyrazolo[1,5-*c*]quinazoline-1-carboxylate derivative discovered in this work acts as a catalytic inhibitor of DNA gyrase and therefore represents a new structural type of catalytic inhibitor of DNA gyrase.

1. Introduction

Fluoroquinolones are potent synthetic antibacterial agents that are used to treat both gram-negative and -positive pathogens. The cellular targets of fluoroquinolones are the bacterial type-II topoisomerases, DNA gyrase (gyrase) and topoisomerase IV (topo IV).[1–4] As for many other antibacterial agents, bacterial resistance is a major issue with fluoroquinolone use. A potential solution to bacterial resistance to fluoroquinolones is the further development of a similar class of antibacterial agents, the quinazoline-2,4-diones.

Bacterial type-II topoisomerases are important for various cellular processes, specifically with DNA replication and transcription.[4–7] An inherent issue of DNA, due to its helical nature, is that positive supercoils accumulate during DNA replication or transcription, which may halt the progression of. Type-II topoisomerases are enzymes that can remove positive supercoils.[4–7] They bind to DNA and cleave two DNA strands four base pairs apart to form a double-strand break. At the site of DNA strand cleavage, an active-site tyrosine residue is covalently attached to the phosphate backbone of DNA. Upon the completion of the catalytic reaction, cleaved DNA strands were re-ligated. Both fluoroquinolone-class and quinazoline-2,4-dione-class antibacterial agents bind to covalent type-II topoisomerase-DNA complexes and form ternary complexes consists of a fluoroquinolone or quinazoline-2,4-dione, a bacterial type-II topoisomerase, and cleaved DNA. Ternary complex formation ultimately "poisons" a bacterial cell by halting DNA replication forks and/or transcription machinery, and generating double-strand breaks. Thus, fluoroquinolones and quinazoline-2,4-diones are often referred to as "topoisomerase poisons."[1–4]

The X-ray crystal structure of the fluoroquinolone (moxifloxacin) in ternary complex with *Acinetobacter Baumannii* topo IV and cleaved DNA (Fig. 1A) displays key interactions between the fluoroquinolone and topo IV and DNA.[8] The interactions include a hydrogen bond between the ribose oxygen of an adenine nucleotide on DNA with a secondary amine nitrogen on the *C-7* pyrrolopyridine moiety of moxifloxacin, and a magnesium-water bridge with the serine and glutamate residues on the ParC subunit of the enzyme and the beta-keto acid moiety on moxifloxacin. These serine and glutamate residues are frequently mutated during quinolone resistance development, highlighting the

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https://doi.org/10.1016/j.bmc.2020.115439
Received 18 October 2019; Received in revised form 4 March 2020; Accepted 11 March 2020
Available online 13 March 2020

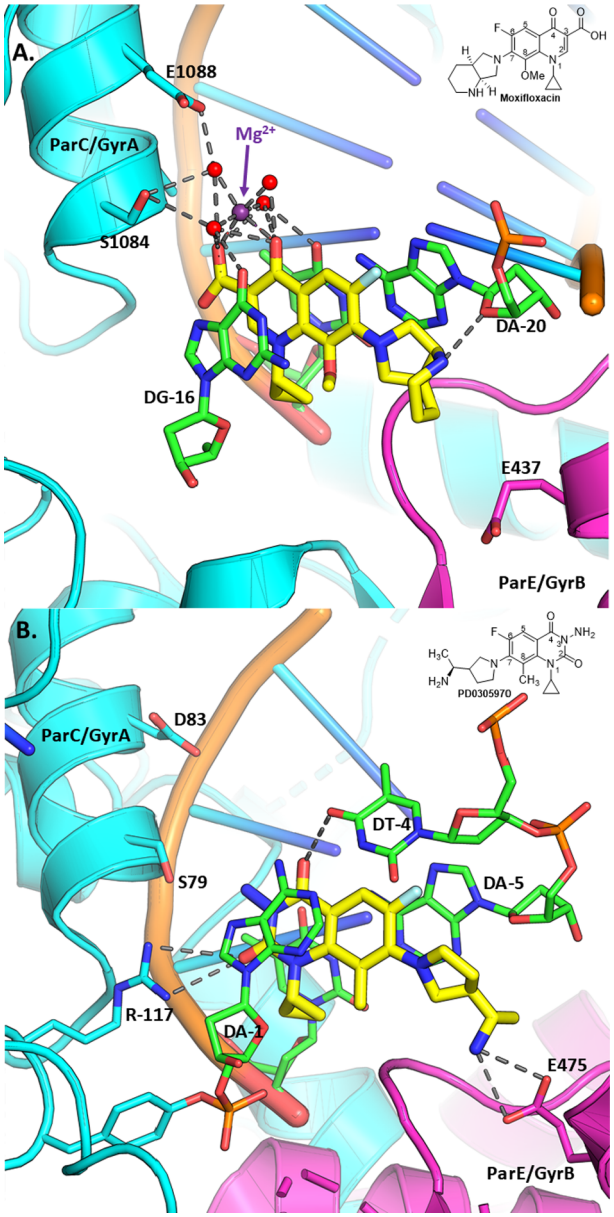


Fig. 1. (A) Crystal structure of moxifloxacin, DNA, and topo IV (from *A. baumannii*). Fluoroquinolone displayed as yellow sticks, water molecules displayed in red, divalent magnesium ion displayed in purple, DNA bases in green, ParC/GyrA in cyan, and ParE/GyrB in magenta. Hydrogen-bond interactions displayed as black dashed lines. Resolution: 3.25 Å. Adapted from RCSB PDB: 2XKK, visualized with T\the PyMOL Molecular Graphics System, Version 2.3.2 (Schrödinger, LLC). Inset figure: Chemical structure of moxifloxacin with core atoms numbered. (B) Crystal structure of PD0305970, DNA, and topo IV (from *S. pneumoniae*). Quinazoline-2,4-dione displayed as yellow sticks, DNA bases in green, ParC/GyrA in cyan, and ParE/GyrB in magenta. Hydrogen-bond interactions displayed as black dashed lines. Resolution: 3.24 Å. Adapted from RCSB PDB: 3RAF, visualized with The PyMOL Molecular Graphics System, Version 2.3.2 Inset figure: Chemical structure of PD0305970 with core atoms numbered.

importance of the magnesium-water bridge.[1–4] The quinazoline-2,4-dione (PD0305970) (Fig. 1B) is also able to form ternary complex with topo IV and DNA.[9] Key interactions between the quinazoline-2,4-dione and topo IV in ternary complex include a hydrogen-bond between the carboxylate moiety of the glutamate residue on the ParE subunit of topo IV and the secondary amine nitrogen on the *C-7* amino-ethyl-pyrrolidine moiety on PD0305970, and hydrogen bond between the arginine residue on the ParC subunit of the enzyme and the *C-2* carbonyl oxygen on PD0305970.

Interactions of both fluoroquinolones and quinazoline-2,4-diones with the topoisomerase and DNA in ternary complexes are similar, however, the distinct difference is the absence of a magnesium-water bridge formation with a quinazoline-2,4-dione (Fig. 1). This difference between fluoroquinolones and quinazoline-2,4-diones affects *in vitro* poisoning activities against the wild-type and fluoroquinolone-resistant *Bacillus anthracis* topo IVs. Topoisomerases with amino acid substitutions for the serine residue involved in magnesium-water bridge formation diminish fluoroquinolone poisoning activity as compared to the wild-type. However, cognate quinazoline-2,4-diones are equipotent with the wild-type and mutant topoisomerases, in terms of poisoning activity.[10] Quinazoline-2,4-diones, nonetheless, display less potent poisoning activity compared to fluoroquinolones.

Further investigation of the ternary complex structure of a quinazoline-2,4-dione with topo IV and DNA (Fig. 1B) identified a region with potential for creating new binding interactions; substitution of functional groups at the *N*-3 position of a quinazoline-2,4-dione might be extended to form direct binding with the helix-4 region of the ParC subunit (Fig. 2). This promoted further exploration of the *N*-3 position on the quinazoline-2,4-dione scaffold to design new quinazoline-2,4-dione derivatives bearing extended primary amine groups at the *N*-3 position, which could directly interact with an amino acid residue(s) on helix-4 of either GyrA or ParC. It was anticipated that a flexible substituent at the *N*-3 position might be able to form different binding contacts with different amino acid residues on helix-4 depending on the amino acid residues present (wild-type vs. quinolone resistant). Furthermore, both fluoroquinolones and quinazoline-2,4-diones form pi-stacking interactions with neighboring DNA bases in the ternary complex,[8,9] which lead to further exploration of quinazoline-2,4-diones having extended ring structures at the *N*-3/*C*-4 position to potentially interact with both the helix-4 region of GyA or ParC and DNA bases in the ternary complex. Modified quinazoline-2,4-diones, either by extending the primary amine at the *N*-3 position or ring extension at the *N*-3/*C*-4 position, were envisioned to serve as initial probes for creating

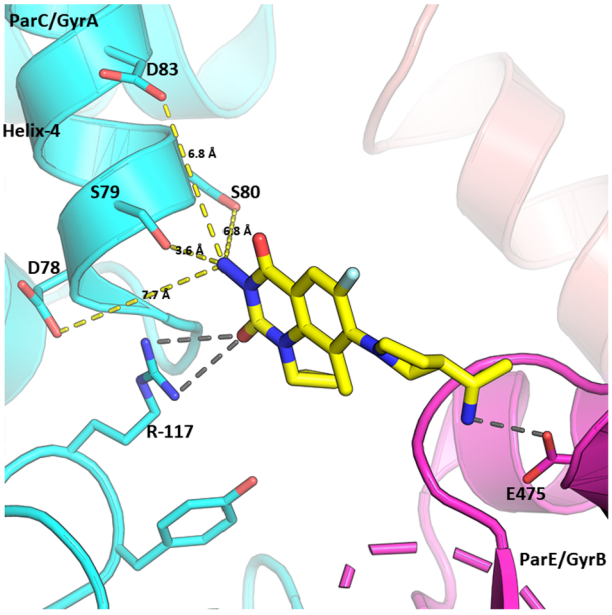


Fig. 2. Potential binding contacts with helix-4 (cyan spiral), containing Ser79, Ser80, Asp83 and Asp78, of *S. pneumonia* ParC. Yellow dashed lines indicate the distance between N3-NH_2 and respective amino acid residues. Existing hydrogen-bond interactions displayed as black dashed lines. Adapted from RCSB PDB: 3RAF, visualized with The PyMOL Molecular Graphics System, Version 2.3.2.

a new interaction(s) between quinazoline-2,4-diones and the helix-4 region of the GyrA/ParC subunit in ternary complex. Such interaction may improve the antibacterial activity of quinazoline-2,4-diones.

There are multiple amino acid residues, one aspartate and at least one serine residue, and also backbone amide bonds, in the helix-4 region of the *S. pneumoniae* ParC subunit that may potentially interact with an *N-3* modified quinazoline-2,4-dione (Fig. 2).[9] The distances between Ser79 and Ser80 hydroxyl groups and the *N*-3-amino group on PD0305970 are 3.6 and 6.8 Å, respectively, and the distance between the Asp78 carboxylic acid group and the *N*-3-amino group on PD0305970 is 7.7 Å.

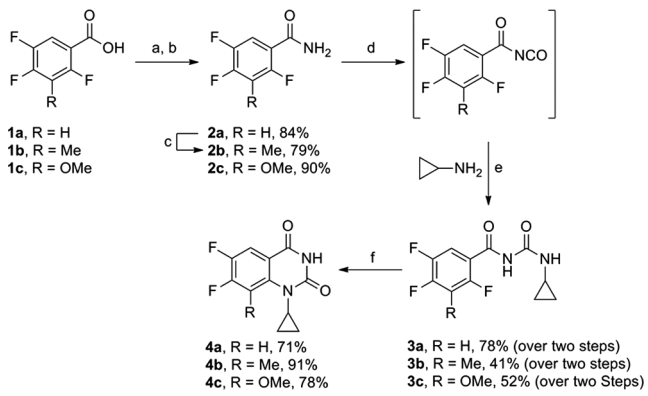
Thus, both serine hydroxyl groups and aspartate carboxylic group are too far for hydrogen-bond interaction between an *N*-3-amino group on quinazoline-2,4-dione. A method to create a hydrogen bond interaction with these residues is to extend the primary amine on the quinazoline-2,4-dione scaffold in order to "close the gap" between the hydroxyl/carboxylic acid groups on the Ser79, Ser80, and Asp78 and the *N*-3-amino group on a quinazoline-2,4-dione. Furthermore, extending the ring system on a quinazoline-2,4-dione is expected to not only increase pi-stacking interaction to neighboring DNA bases, but also introduce a new carboxylic ester/acid moiety on the quinazoline-2,4-dione scaffold to further probe the helix-4 region of the bacterial type-II topoisomerase.

2. Results and discussion

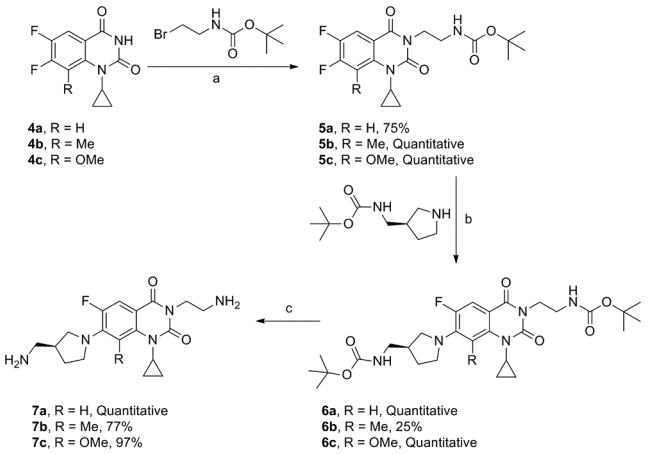
2.1. Chemistry

N-3/*C*-4 modified quinazoline-2,4-diones were synthesized as follows. Synthesis of the dione core (Scheme 1) was adapted from Tran et al.,[11] Commercially available trifluorobenzoic acids **1a** and **1c** were converted to their corresponding amides via formation of the acid chloride with oxalyl chloride followed by ammonium hydroxide treatment. Amide **2a** was treated with a non-nucleophilic base and consequently methyl iodide to form the methyl substituted trifluorobenzamide **2b**. The amides **2a-c** were then converted to their corresponding cyclopropylamine substituted ureas via formation of isocyanate intermediate followed by treatment with a primary amine at low temperatures. Ureas **3a-c** were then cyclized to the corresponding dione cores **4a-c** under basic conditions.

Each of the *N3*-H quinazoline-2,4-diones **4a-c** was substituted at N3 with Boc-protected 2-bromoethylamine via Sn2 substitution under basic conditions to give the Boc-protected ethylamine diones **5a-c**. The protected ethylamine diones **5a-c** were then substituted at C7 with a secondary amine via SnAr substitution under basic conditions, the resulting diones **6a-c** were then subject to acid-mediated removal of Boc



Scheme 1. Synthesis of the *N-3*–H Quinazoline-2,4-dione cores **4a-c**. Reagents and conditions: (a) $(COCl)_2$, DCM/DMF, RT, 2 h; (b) NH_4OH, DCM, 0 °C – RT, 15 min; (c) i. LiHMDS, THF, −78 °C, 20 min; ii. MeI, THF, 0 °C – RT, 3.5 h; (d) $(COCl)_2$, DCM, 90 °C, 4 h; (e) p-dioxane, 0 °C, 18–19 h; (f) i. KHMDS, THF, −10 °C – RT, 10 min; ii. 18-crown-6, THF, 105 °C, 1.5–2.5 h.

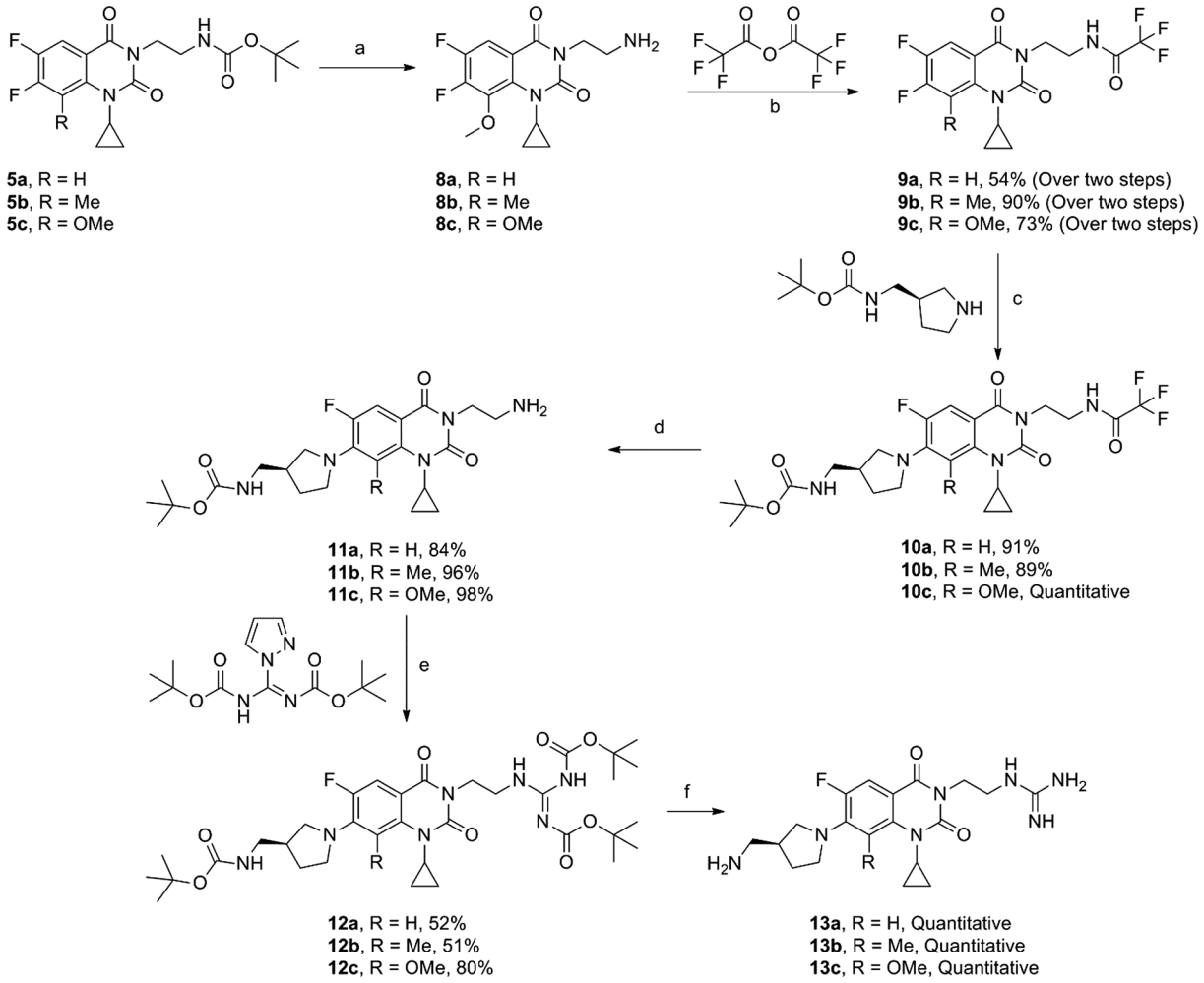


Scheme 2. Synthesis of *N-3*-ethylamine Quinazoline-2,4-diones **7a-c**. (a) K_2CO_3, DMF, RT, 19–26 h; (b) TEA, acetonitrile, 95 °C, 26–168 h; (c) HCl, acetonitrile: H_2O, RT, 22–23 h.

groups to obtain the target diones **7a-c** (UIAA-I-133, UIAA-I-227, UIAA-I-131) (Scheme 2).

Synthesis of quinazoline-2,4-diones (Scheme 3) substituted with an alkyl guanidine was adapted from Bernatowicz et al.,[12] Beginning from the previously synthesized protected ethylamine diones **5a-c**, the acid-mediated removal of the Boc group was achieved resulting in the ethylamines **8a-c**. Protection of the primary amines **8a-c** as trifluoracetamides was achieved using trifluoroacetic anhydride in the presence of base under mild conditions to produce diones **9a-c**. SnAr substitution of Boc-protected aminomethylpyrrolidine at C7 afforded orthogonally protected diones **10a-c**. Selective cleavage of the trifluoroacetamides provided diones with unsubstituted primary amine **11a-c**. Guanylation of the primary amine provided tris-Boc-protected ethylguanidine diones, **12a-c**, which were consequently deprotected under acidic conditions to produce the desired N3 ethylguanidine modified quinazoline-2,4-diones, **13a-c** (UIAA-II-080, UIAA-II-082, UIAA-II-079).

The synthesis of the ethyl 5,6-dihydropyrazolo[1,5-*c*]quinazoline carboxylates proved to be efficient (6 steps) using a new approach to quinazoline-2,4-dione syntheses (Schemes 4 and 5**)**. The synthesis begins with a commercially available difluoroindoline-2,3-dione, **14**, which is substituted with a p-toluenesulfonyl protected hydrazine under heat to produce the corresponding p-toluenesulfonyl-hydrazone, which is immediately oxidized in the presence of sodium hydroxide to the corresponding 3-diazo-1,3-difluoroindoline, **15**, (adapted from Marti *et al.*).[13] The diazooxindole, **15**, was cyclized with ethyl propiolate in a [3 + 2] fashion under reflux to yield the desired ethyl 5,6-dihydropyrazolo[1,5-*c*]quinazoline-2-carboxylate **16a** and the undesired regioisomer (ethyl 5,6-dihydropyrazolo[1,5-*c*]quinazoline-1-carboxylate) **16b** in a 4:1 isomeric ratio, respectively. This cyclization step was adapted from Vogt et al.,[14] which claimed efficient cyclization of diazooxindoles to their corresponding tricyclic esters, however, no reaction mechanisms were proposed or cited. Moreover, a reaction mechanism is proposed and rationalized via a modified 1,3-dipolar cycloaddition route, where the diazooxindole is the dipole and the ethyl propiolate is the dipolarophile (Fig. 3). Explanation of the two regioisomers and their respective ratios is further explained using frontier molecular orbital (FMO) theories (Fig. 4); Sustmann and coworkers' early work on cycloadditions has proven that diazomethanes react predominantly via $HOMO_{1,3\text{-dipole}} - LUMO_{dipolarophile}$ bonding interaction, however, the opposite bonding interaction – $LUMO_{1,3\text{-dipole}} - HOMO_{dipolarophile}$ – is also observed.[15,16] Padwa and coworkers displayed a very similar result in their work with carbonyl ylides (1,3-dipoles) and methyl propiolates (dipolarophile), and observed a regioisomeric ratio between $HOMO_{1,3\text{-dipole}} - LUMO_{dipolarophile}$ and



Scheme 3. Synthesis of the *N-3*-ethyl guanidine Quinazoline-2,4-diones **13a-c**. (a) DCM:TFA, RT, 14–19 h (b) TEA, $CHCl_3$, 0 °C – RT, 16–48 h; (c) TEA, DMSO, 55 °C, 16–96 h; (d) NH_4OH, MeOH, RT, 14 h; (e) $CHCl_3$, 40 °C, 72 h; (f) HCl, acetonitrile: H_2O, RT, 10 h.

$LUMO_{1,3\text{-dipole}}$ – $HOMO_{dipolarophile}$ of 4:1, respectively.[17]

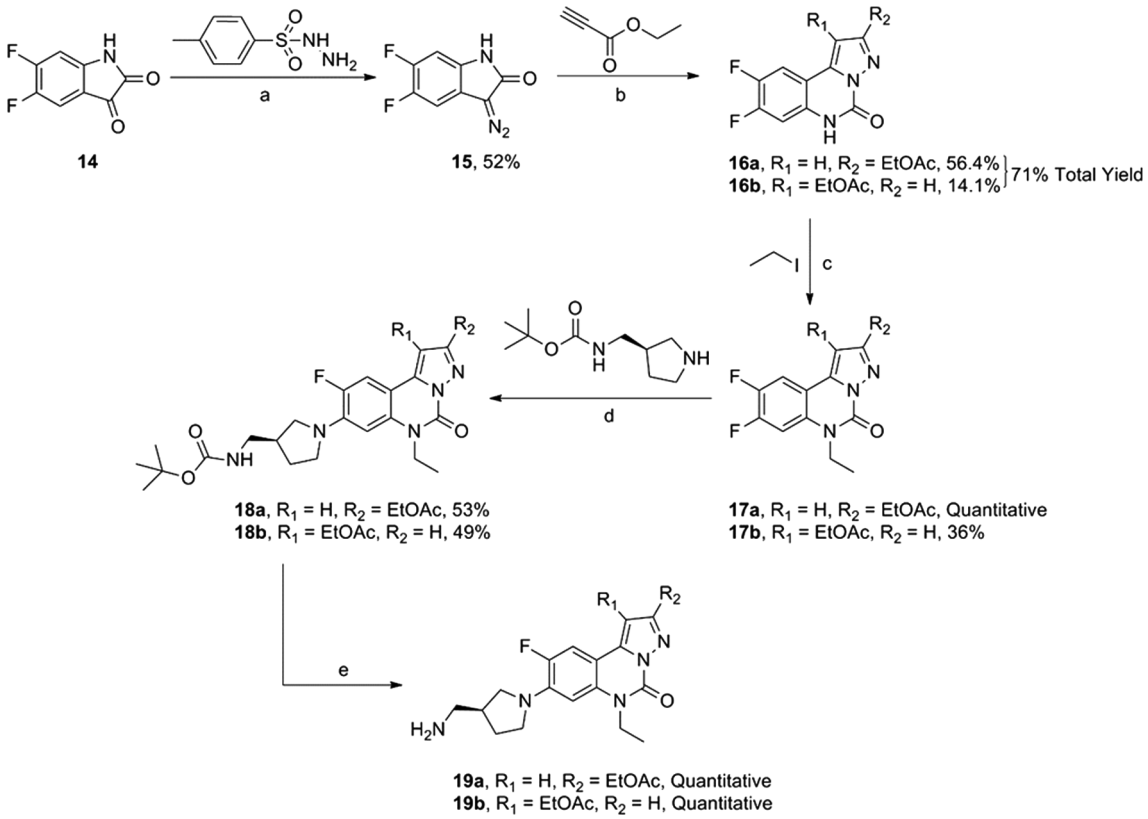
These literature findings further corroborate the observed ethyl 5,6-dihydropyrazolo[1,5-*c*]quinazoline carboxylates, **16a-b**, and their proposed reaction mechanisms. Nevertheless, the mixture of isomers, **16a-b**, afforded two ethyl 5,6-dihydropyrazolo[1,5-*c*]quinazoline carboxylates, which were then *N1* alkylated with ethyl iodide, under basic conditions, to afford the consequent *N1*-ethyl substituted ethyl 5,6-dihydropyrazolo[1,5-*c*]quinazoline carboxylates, **17a-b**, in a similar 4:1 ratio, which were readily purified and isolated via preparative-HPLC. SnAr substitution of *C7* positions of the ethyl 5,6-dihydropyrazolo[1,5-*c*]quinazoline-1-carboxylate **17a-b** afforded **18a-b**, which were then treated with acid to remove Boc protecting groups to afford the ethyl 5,6-dihydropyrazolo[1,5-*c*]quinazoline carboxylates, **19a-b** (UIAA-II-223 and -232). Additionally, **18a** was subjected to base-catalyzed ester hydrolysis and acid-mediated removal of Boc group to afford the corresponding pyrazole-fused dione carboxy-acid, **20** (UIAA-II-226) (Scheme 5).

2.2. Biochemical evaluation

Escherichia coli DNA gyrase was used as a model bacterial type-II topoisomerase to assess the activities of diones. The supercoiling and DNA cleavage assays were performed as described previously.[18] First, supercoiling assays were performed in the presence of 10, 25, or 50 μM of *N-3/C-4* modified quinazoline-2,4-diones, **7a-c**, **13a-c**, **19a-b**, and **21** to identify inhibitory compounds. Only **7b**, **13b**, and **19b** exhibited significant inhibition at 25 and 50 μM (Fig. S1). Additional inhibition of supercoiling activities was determined with these three compounds to obtain IC_{50} values (the concentration required to inhibit 50% of DNA gyrase-catalyzed supercoiling activity). The IC_{50} values of **7b**, **13b**, and **19b** were significantly lower than those of a control fluoroquinolone, UING-V-249, and a control quinazoline-2,4-dione, UIJR-I-048 (Table 1).

Of the *N*-3-alkylamine quinazoline-2,4-diones (**7a-c**) evaluated, only **7b** displayed modest supercoiling inhibition activity against DNA gyrase (Table 1), with the only difference between each compound is their respective substituents on the *C*-8 positions of the quinazoline-2,4-dione core. The inhibitory effect of these compounds was decreased compared to control compounds, UING-V-249 and UIJR-I-048. Possible explanations in the decrease in supercoiling inhibition is the overall entropic penalty of adding a flexible linker in-between the *N*-3 amine and the quinazoline-2,4-dione core, instead of a rigid linker.[19]

Of the *N*-3-alkylguanidine quinazoline-2,4-diones (**13a-c**) evaluated, only **13b** displayed supercoiling inhibition activity against DNA gyrase (Table 1), again with the C8-methyl substituent providing the highest potency, which has been observed in previous studies with C8-methyl substituted fluoroquinolones and quinazoline-2,4-diones.[20] There is a slight increase in potency of the *N*-3-alkylguanidine quinazoline-2,4-dione, **13b**, over the *N-3*-alkylamine quinazoline-2,4-dione, **7b** (Table 1); a possible explanation for this outcome is the slight extension of the primary amine in **13b**, compared to **7b**, which may form a more favorable interaction in the helix-4 region of the GyrA/ParC subunit in ternary complex.



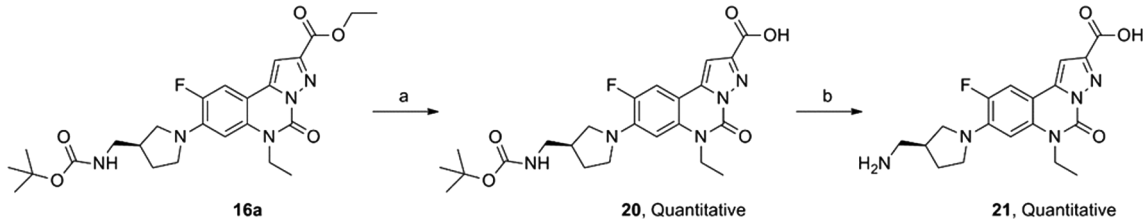
Scheme 4. Synthesis of the ethyl 5,6-dihydropyrazolo[1,5-*c*]quinazoline carboxylate**s 19a** and **19b**. (a) i. MeOH, 60 °C, 24 h; ii. NaOH, 64 °C, 4 h; (b) toluene, 115 °C, 17 h; (c) DIPEA, DMSO, 40 °C, 16–41 h; (d) TEA or DIPEA, DMSO, 60 °C, 18–20 h; (e) HCl, acetonitrile: H_2O, RT, 4 h.

Lastly, of the ethyl 5,6-dihydropyrazolo[1,5-*c*]quinazoline carboxylates/carboxylic acid (**19a-b, 20**) evaluated, only **19b** displayed supercoiling inhibition activity against DNA gyrase and was the most potent compound among those tested in this study. However, its activity was still lower than that of either the control fluoroquinolone or quinazoline-2,4-dione (Table 1).

We also performed DNA cleavage assays with **7b**, **13b**, and **19b** to determine if these compounds poison DNA gyrase. When a fluoroquinolone or quinazoline-2,4-dione poisons DNA gyrase, a cleaved duplex is trapped in the ternary complex. In the DNA cleavage assay where DNA gyrase in the ternary complex is denatured and removed, the ternary complex is converted into a double-strand break. Thus, the poisoning of DNA gyrase is measured as the generation of the linear plasmid DNA. In our previous study,[21,22] we determined the CC_3 value (the concentration required to triple the level of the linear DNA from the background level of the linear DNA in the absence of drug) against DNA gyrase of each compound to compare the effectiveness of poisoning. Normally, the CC_3 values of fluoroquinolones and quinazoline-2,4-diones, such as UING-V-249 and UIJR-I-048, are 10-fold lower than the IC_{50} values determined in the supercoiling assay.[21,22] However, we were able to detect increases in the level of linear DNA only at 25–50 μM (Table 1, Fig. S2). Thus, **7b** and **13b** could poison DNA gyrase but not do so as efficiently as fluoroquinolones and quinazoline-2,4-diones. Unexpectedly, no poisoning effect was observed with **19b**, suggesting that **19b** acts as a gyrase catalytic inhibitor, not as a gyrase poison.

We examined if **19b** could bind to DNA directly in the absence of any topoisomerase; a potent DNA binder could inhibit the supercoiling activity of DNA gyrase by preventing its binding to DNA. A direct fluorescence-based DNA binding assay[23] showed that **19b** did not bind directly to DNA alone (supplemental material Fig. S3). A thiazole orange displacement assay[23] further demonstrated that **19b** did not intercalate or otherwise bind DNA and interfere with intercalator binding.

Toward conclusion of this study, the imidazopyrazinones (IPYs) with antibacterial activity have been reported.[24,25] Tricyclic IPYs, the most potent IPYs contain the quinazolinedione and thus there are structural similarities between tricyclic IPYs and our compounds, especially the ethyl 5,6-dihydropyrazolo[1,5-*c*]quinazoline carboxylates. Biochemical and structural studies have shown that tricyclic IPYs bind to a quinolone binding pocket and poison DNA gyrase.[24] Unlike fluoroquinolones, they do not require the water–metal–ion bridge for their binding. The docking of **19b** to the imidazopyrazinone T3 binding site in *S. **aureus*** gyrase-T3-DNA the ternary complex (RPD



Scheme 5. Synthesis of the 5,6-dihydropyrazolo[1,5-*c*]quinazoline-2-carboxylic acid **21**. (a) NaOH (aq.), RT, 4 h; (b) HCl, acetonitrile: H_2O, RT, 2 h.

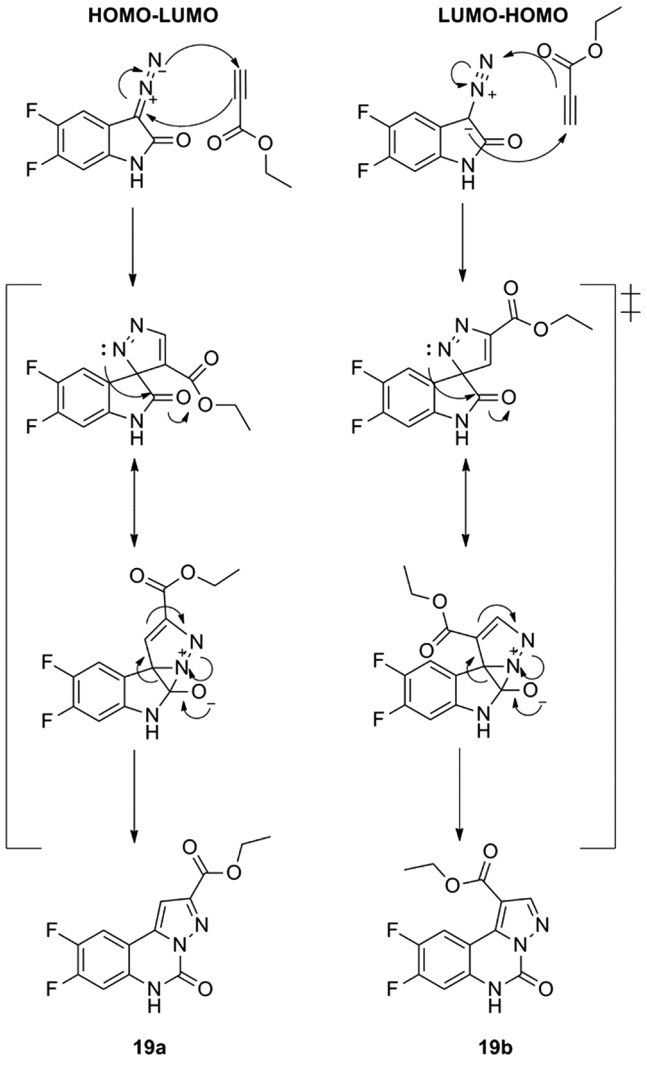


Fig. 3. Proposed 1,3-cycloaddition mechanism of the ethyl 5,6-dihydropyrazolo [1,5-*c*]quinazoline carboxylates, **19a-b**.

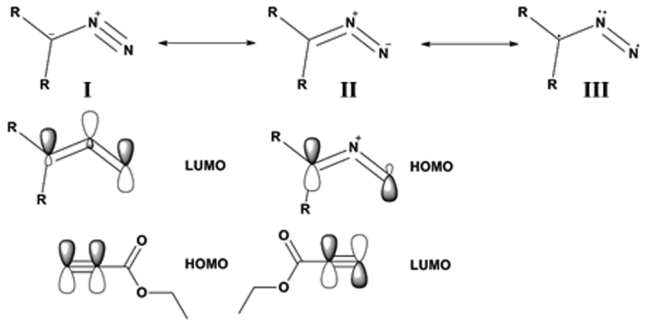


Fig. 4. Diazooxindole resonance structures and the corresponding 1,3-dipole and dipolarophile HOMO and LUMO states.

ID: 6FQS)[24] showed that **19b** may bind to a DNA gyrase-DNA complex in the traditional mode, but not an alternate mode, of T3 binding (data not shown). Thus, **19b** and T3 bind to a DNA gyrase-DNA complex in similar but distinctive manners. Both the study on IPYs and this work support the possibility that quinazolinedione-like compounds may be developed as novel DNA gyrase inhibitors. Subtle differences in chemical structures and interactions between quinazolinedione-like compounds and a DNA gyrase-DNA complex appear to determine if each particular compound acts as a DNA gyrase poison or as a catalytic inhibitor of DNA gyrase.

3. Conclusion

In conclusion, guided by x-ray crystal structures of both fluoroquinolone- and quinazoline-2,4-dione-bound topoisomerase-DNA ternary complex, novel *N*-3/*C*-4 quinazoline-2,4-diones and dione-like compounds, which may directly bind to an amino acid residue(s) in the helix-4 region of the GyrA/ParC subunit, were designed and synthesized. Activities of *N*-3/*C*-4 quinazoline-2,4-diones suggest that, unlike the typical *N*-3 amine quinazoline-2,4-dione, such as UIJR-I-048, *N*-3 alkylamine/alkylguanidine quinazoline-2,4-diones cannot poison DNA gyrase. The ethyl 5,6-dihydropyrazolo[1,5-*c*]quinazoline-1-carboxylate (**19b**) displayed the most potent supercoiling inhibition activity among the compounds tested in this study but it did not poison DNA gyrase. Therefore, **19b** acts as a catalytic inhibitor of DNA gyrase. Further studies are underway to determine the mechanism of **19b**. **19b**, as well as **7b** and **13b**, represent novel chemical structures for gyrase inhibitors. Identification of their derivatives with higher potency may lead to the development of new gyrase inhibitors, catalytic inhibitors, and/or poisons. Such gyrase inhibitors may be effective against fluoroquinolone-resistant mutant gyrases and topo IVs.

4. Materials and methods

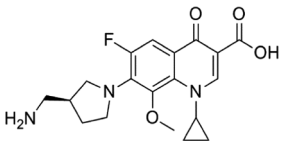
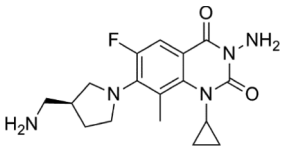
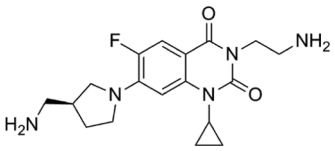
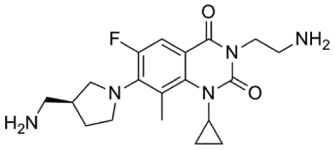
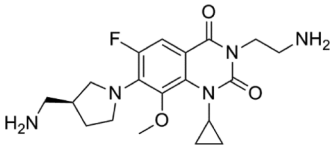
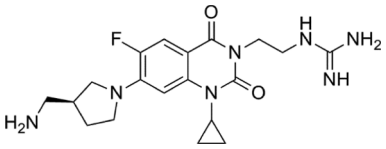
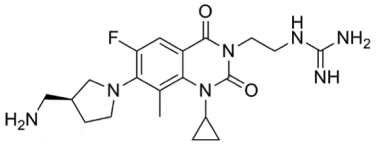
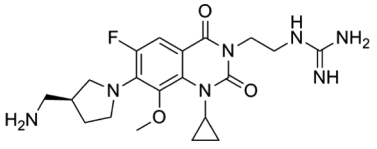
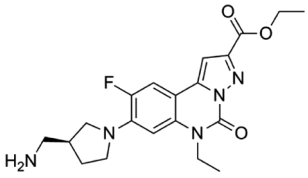
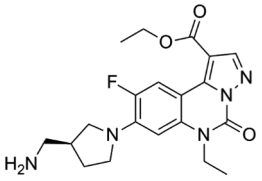
4.1. Calf thymus DNA (ct-DNA) binding assay

The purity and concentration of ct-DNA, purchased from Worthington Biochemical Corporation, was determined by absorption ratio A260/280 analysis, utilizing the BioTek Synergy 2 Multi-Detection Microplate reader and Gen5 2.0 software. The attenuance ratio averaged at 1.88. The molar concentration of Ct-DNA was determined by Beer's Law (A = εbc, where ε = the molar extinction coefficient, b = path length, and c = molar concentration); the molar extinction coefficient for Ct-DNA is 6600 M^{-1} cm^{-1} at 260 nm per nucleotide and the path length is 0.05 cm. The binding ability of both **27b** and moxifloxacin was measured by the innate fluorescence, which was determined in the absence of DNA on a Perkin Elmer EnVision multi-label plate reader, utilizing COSTAR clear cell culture plates. All DNA incubations with test compound (dissolved at 1000× in DMSO) were at a final volume of 100 μL. The pyrazole-fused tricyclic dione, **27b**, and moxifloxacin concentration were kept constant at 25 μM, while the Ct-DNA molar concentration ranged from 0 to 100 μM. The dione, **27b**, and Moxifloxacin were incubated in 10 mM Tris-HCl buffer (pH = 7.4) for 10 min, at room temperature, before fluorescence measurement. 100% fluorescence = absence of ct-DNA in incubation.

4.2. General chemistry methods

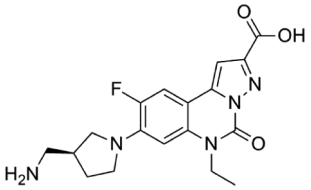
Mass spectrometry data were collected with a Waters Q-TOF Premier mass spectrometer, utilizing a direct flow injection for 1 min and ESI-positive ionization. ^{1}H and ^{19}F NMR spectra were collected using a Bruker Ultrashield 300 MHz instrument. Chemical shifts are reported as ppm relative to tetramethylsilane (TMS) and deuterated solvent ($CDCl_3$ or DMSO-d_6, unless stated otherwise). Retention time was determined by analytical HPLC utilizing a Shimadzu HPLC system, comprised of a Nexera X2 SPD-M30A diode array detector, CBM-20A system controller, LC-20AT pump, and DGU-14A solvent degasser. HPLC was operated with Shimadzu LabSolutions V.5.85 software on a Dell OptiPlex 5040 PC. The HPLC columns used were Restek Allure PFP Propyl (5μ; 4.6 × 150 mm) or a Phenomenex Luna 5u C18(2) 100A (5μ; 4.6 × 250 mm). Mobile phase A consisted of water with 0.1% trifluoroacetic acid; mobile phase B consisted of acetonitrile with 0.1% trifluoroacetic acid. The HPLC method utilized for analyses was a gradient elution from 5% B to 95% B over 30 min with a flow rate of

Table 1
DNA gyrase supercoiling inhibition and DNA gyrase poisoning activities of modified *N*-3/*C*-4 quinazoline-2,4-diones and dione-like compounds.

Compound #	Compound ID	Structure	Supercoiling Inhibition IC_{50} (μM)	Poisoning Activity
–	UING-V-249[1]		0.12 ± 0.01	Yes
–	UIJR-I-048[2]		0.95 ± 0.15	Yes
7a	UIAA-I-133		> 50	N/D
7b	UIAA-I-227		17.6 ± 0.8	Yes
7c	UIAA-I-131		> 50	N/D
13a	UIAA-II-080		> 50	N/D
13b	UIAA-II-082		11.5 ± 0.3	Yes
13c	UIAA-II-079		> 50	N/D
19a	**UIAA-II-223**		> **50**	**N/D**
19b	UIAA-II-232		3.5 ± 0.1	No

(*continued on next page*)

Table 1 (*continued*)

Compound #	Compound ID	Structure	Supercoiling Inhibition IC_{50} (μM)	Poisoning Activity
21	UIAA-II-226		> 50	N/D

[1] Control fluoroquinolone.
[2] Control quinazoline-2,4-dione.

1.000 mL/min. Final products were either purified via flash chromatography, utilizing Silica Gel 60 (particle size 0.040 – 0.063 mm; 230–400 mesh ASTM) with multiple mobile phase systems or a Shimadzu preparative HPLC system, comprised of a SPD20A UV/Vis detector, LC-20AP pump, and FCV-200AL Prep Quaternary valve. Preparative HPLC was operated with Shimadzu LabSolutions V.5.85 software on a Dell OptiPlex 9020 PC. The HPLC column used was a Phenomenex Luna 10u C18 100A (10 μ; 21.2 × 250 mm). Mobile phase A consisted of water with 0.1% trifluoroacetic acid; mobile phase B consisted of acetonitrile with 0.1% trifluoroacetic acid. The preparative HPLC method utilized for purification was a gradient elution from 5% B to 95% B over 60 min with a flow rate of 10.000 mL/min.

4.3. Scheme 1. Synthesis of N-3-H Quinazoline-2,4-dione cores, **4a-c**

4.3.1. **Preparation of 2,4,5-trifluorobenzamide (2a) (UIAA-I-061)**

Commercially available 2,4,5-trifluorobenzoic acid (**1a**) (10.1 g, 57.4 mmol) was dissolved in 100 mL of distilled DCM and stirred under argon atmosphere. Oxalyl chloride (5.0 mL, 58.300 mmol) was then added to the stirring mixture, the solution was allowed to stir for 5 min at room temperature. Anhydrous DMF (0.45 mL, 5.8 mmol) was added, dropwise, to the stirring solution over 35 s, the resulting solution was allowed to stir at room temperature for 2 h. The DCM was removed from the reaction mixture by rotary evaporation, the resulting oil was reconstituted in 100 mL of DCM and placed in an ice bath to cool to ~ 0 °C. Ammonium hydroxide (40 mL, 28–30% ammonia) was added, dropwise, to the cool stirring solution over 2 min, the resulting mixture was allowed to stir for 2.5 h, gradually warming up to room temperature. The product was extracted by washing the aqueous layer with 100 mL of DCM and then 150 mL of ethyl acetate. The organic layers were pooled and concentrated by rotary evaporation, the resulting solid was triturated with 9:1 hexanes:ethyl acetate and subjected to vacuum filtration, which yielded **UIAA-I-061** (**2a**) as a pure yellow solid. 84% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.01 (ddd, J = 7.17, 8.94, 10.62 Hz, 1H), 7.05 (ddd, J = 6.05, 9.51, 10.83 Hz, 1H), 6.66 (bs, 1H), 6.03 (bs, 1H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −113.67 (m, 1F), −125.90 (ddd, J = 9.08, 17.12, 21.57 Hz, 1F), −140.15 (m, 1F). HRMS (ESI) calculated for (M + H^+) 176.0318, found 176.0323. Retention time (analytical HPLC) = 12.3 min.

4.3.2. Preparation of 2,4,5-trifluoro-3-methylbenzamide (2b) (UIAA-I-053)

To a stirring solution of **UIAA-I-061** (**2a**) (0.725 g, 4.14 mmol) in 15 mL of distilled THF, LiHMDS (1.0 M in THF, 21 mL, 21 mmol) was added, dropwise, through an addition funnel at 0 °C over 30 min and under argon atmosphere; the resulting mixture was allowed to stir for 3.5 h at 0 °C. Iodomethane (0.7 mL, 11.2 mmol) was then added to the cool reaction mixture and was stirred for an additional 3.5 h at 0 °C. The resulting reaction mixture was quenched with 50 mL of 1 N HCl and the organic layer was extracted with 30 mL of ethyl acetate and washed twice with 30 mL of sodium bisulfite (625 mg/mL) and then with 30 mL of distilled water. The organic layers were pooled, filtered through Na_2SO_4, and concentrated via rotary evaporation; the resulting yellow residue was recrystallized with 9:1 hexanes:ethyl acetate, to afford pure **UIAA-I-053** (**2b**) as a yellow crystalline solid. 79% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.83 (dd, J = 9.45, 17.36 Hz, 1H), 6.68 (bs, 1H), 5.97 (bs, 1H), 2.31 (t, J = 2.26 Hz, 3H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −117.75 (s, 1F), −129.79 (m, 1F), −140.69 (ddd, J = 10.61, 16.39, 21.77 Hz, 1F). HRMS (ESI) calculated for (M + H^+) 190.1419, found 190.0483. Retention time (analytical HPLC) = 14.4 min.

4.3.3. Preparation of 2,4,5-trifluoro-3-methoxybenzamide (2c) (UIAA-I-046)

Commercially available 2,4,5-trifluorbenzoic acid (**1c**) (1.01 g, 4.9 mmol) was dissolved in 7 mL of distilled DCM and stirred under argon atmosphere. Oxalyl chloride (0.6 mL, 7.0 mmol) was then added and allowed to stir 5 min at room temperature. Anhydrous DMF (0.080 mL, 1.033 mmol) was added to the stirring mixture, dropwise, the reaction mixture was then allowed to stir at room temperature for 2 h. The DCM was removed by rotary evaporation and reconstituted with 8 mL of distilled DCM and cooled to ~ 0 °C. Ammonium hydroxide (5 mL, 28–30% ammonia) was added, dropwise, to the cool stirring solution over 2 min, the resulting mixture was allowed to warm up to room temperature, gradually, over 30 min. The resulting reaction mixture was washed with 10 mL of distilled water and the organic layer was collected. The aqueous layer was washed with 3 mL of DCM and 3 mL of ethyl acetate. All the organic layers were pooled and concentrated by rotary evaporation to afford pure **UIAA-I-046** (**2c**) as a pure solid. 90% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.68 (ddd, J = 6.72, 8.62, 10.64 Hz, 1H), 6.61 (s, 1H), 6.05 (s, 1H), 4.08 (s, 3H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −132.80 (s, 1F), −138.31 (ddd, J = 10.71, 13.75, 21.05 Hz, 1F), −143.70 (m, 1F). HRMS (ESI) calculated for (M + H^+) 206.0423, found 206.0434. Retention time (analytical HPLC) = 14.0 min.

4.3.4. Preparation of N-(cyclopropylcarbomoyl)-2,4,5-trifluorobenzamide (3a) (UIAA-I-069)

To a stirring solution of **UIAA-I-061** (**2a**) (0.205 g, 1.170 mmol) dissolved in 4 mL 1,2-dichloroethane, oxalyl chloride (0.145 mL, 1.691 mmol) was added, dropwise, at room temperature and under argon atmosphere. The resulting mixture was then refluxed at 90 °C for 4 h. The reaction mixture was then allowed to cool and concentrated by rotary evaporation to form the reactive isocyanate, *in situ*. The dried isocyanate was then reconstituted in 4 mL of p-dioxane and cooled to 0 °C under argon atmosphere. Cyclopropyl amine (0.096 mL, 1.386 mmol) was then added to the cool solution and the resulting mixture was warmed to room temperature over 18.5 h. The reaction mixture was then concentrated by rotary evaporation and reconstituted in 10 mL of DCM and washed with 30 mL of saturated $NaHCO_3$, 30 mL of distilled water, and 30 mL of saturated brine solution. The organic layer was collected, filtered through Na_2SO_4 and concentrated via rotary evaporation, the resulting solid was triturated with 1:1 ethyl ether: hexanes to afford pure **UIAA-I-069** (**3a**). 78% yield. ^{1}H NMR

(300 MHz,$CDCl_3$) δ = 8.53 (m, 2H), 7.91 (ddd, J = 7.03, 8.71, 10.24 Hz, 1H), 7.10 (ddd, J = 5.95, 9.33, 10.90 Hz, 1H), 2.80 (m, 1H), 0.85 (td, J = 5.18, 7.09 Hz, 2H), 0.66 (m, 2H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −112.29 (s, 1F), −123.17 (dd, J = 10.71, 19.66 Hz, 1F), −138.97 (m, 1F). HRMS (ESI) calculated for (M + Na^+) 281.0508, found 281.0522. Retention factor (TLC) = 0.64, 1:1 ethyl acetate: hexanes.

4.3.5. Preparation of N-(cyclopropylcarbomoyl)-2,4,5-trifluoro-3-methylbenzamide (3b) (UIAA-I-101)

To a stirring solution of **UIAA-I-095** (**2b**) (0.605 g, 3.199 mmol) in 9 mL of 1,2-dichloroethane, oxalyl chloride (0.420 mL, 4.897 mmol) was added, dropwise, at room temperature and under argon atmosphere. The resulting mixture was refluxed at 95 °C for 4 h. The reaction mixture was allowed to cool and concentrated by rotary evaporation to form the reactive isocyanate, *in situ*. The dried isocyanate was reconstituted in 11 mL p-dioxane and cooled to 0 °C. Cyclopropyl amine (0.340 mL, 4.907 mmol) was then added to the cool solution and the resulting mixture was allowed to slowly warm to room temperature over 18 h. The yellow solution was then concentrated by rotary evaporation and the resulting residue was reconstituted in 30 mL of DCM and subsequently washed with 30 mL of saturated $NaHCO_3$, 30 mL of distilled water, and 30 mL of saturated brine solution. The organic layer was collected, filtered through Na_2SO_4 and concentrated via rotary evaporation, the resulting solid was triturated with 1:1 ethyl ether: hexanes to afford crude **UIAA-I-101 (3b)**, the filtrate was reprocessed to give 2nd crop. The crops were combined and purified via silica gel flash chromatography using a gradient elution from 1:1 to 3:1 ethyl acetate: hexanes with 0.1% (v/v) triethylamine to afford pure **UIAA-I-101 (3b)**. 41% yield. ^{1}H NMR (300 MHz, Acetone) δ = 9.43 (bs, 1H), 8.35 (bs, 1H), 7.61 (m, 1H), 2.76 (qd, J = 3.83, 7.22 Hz, 1H), 2.29 (t, J = 2.05 Hz, 3H), 0.76 (dq, J = 4.70, 6.02 Hz, 2H), 0.60 (td, J = 4.70, 7.26 Hz, 2H). ^{19}F NMR (282 MHz, Acetone) δ = −118.40 (s, 1F), −132.83 (s, 1F), −143.45 (m, 1F). HRMS (ESI) calculated for (M + Na^+) 295.0665, found 295.0674. Retention factor (TLC) = 0.81, 1:1 ethyl acetate: hexanes.

4.3.6. Preparation of N-(cyclopropylcarbomoyl)-2,4,5-trifluoro-3-methoxybenzamide (3c) (UIAA-I-077)

To a stirring solution of **UIAA-I-046** (**2c**) (2.05 g, 9.99 mmol) in 98 mL of distilled DCM, oxalyl chloride (0.900 mL, 10.494 mmol) was added, dropwise, at room temperature and under argon atmosphere. The resulting mixture was refluxed at 60 °C for 19 h. The reaction mixture was allowed to cool and concentrated by rotary evaporation to form the reactive isocyanate, *in situ*. The dried isocyanate was reconstituted in 10 mL of distilled DCM and added, dropwise, to a cool solution, 0 °C, containing cyclopropyl amine (1.0 mL, 14.4 mmol) and 20 mL of distilled DCM. The resulting mixture was allowed to stir at room temperature for 1 h. The yellow solution was then concentrated by rotary evaporation and the resulting residue was precipitated with 1:1 isopropyl alcohol:hexanes solution and filtered to afford **UIAA-I-077** (**3c**) as a pure white solid. 52% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.44 (bs, 2H), 7.56 (dd, J = 8.48, 16.85 Hz, 1H), 4.10 (s, 3H), 2.80 (m, 1H), 0.86 (dd, J = 7.02, 12.46 Hz, 2H), 0.67 (d, J = 7.38, 2H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −131.57 (s, 1F), −137.18 (s, 1F), −141.32 (s, 1F). HRMS (ESI) calculated for (M + Na^+) 311.0614, found 311.0612. Retention factor (TLC) = 0.63, 1:1 ethyl acetate:hexanes.

4.3.7. Preparation of 1-cyclopropyl-6,7-difluoroquinazoline-2,4(1H,3H)-dione (4a) (UIAA-I-091)

To a stirring solution of **UIAA-I-069** (**3a**) (0.530 g, 2.053 mmol) dissolved in 36.5 mL of distilled THF cooled to −10 °C, KHMDS (1.0 M in THF, 4.4 mL, 4.4 mmol) was added, dropwise, under argon atmosphere. The resulting mixture was then allowed to gradually warm to room temperature over 10 min. A catalytic amount of 18-crown-6 (0.109 g, 0.412 mmol) was added in one portion to the room temperature mixture. The reaction mixture was then heated to reflux at 105 °C for 2.5 h. After cooling the hot reaction mixture to room temperature, it was diluted with 30 mL of ethyl acetate and washed/quenched with 80 mL 1 N HCl, 80 mL of distilled water, and finally with 80 mL of saturated brine solution. The organic layers were pooled, filtered through Na_2SO_4, and concentrated by rotary evaporation. The residue was triturated with a 1:1 ethyl ether:hexanes solution and then filtered to afford pure **UIAA-I-091** (**4a**), the filtrate was reprocessed to afford a 2nd crop. 71% yield. ^{1}H NMR (300 MHz, DMSO) δ = 11.61 (bs, 1H), 7.91 (dd, J = 8.84, 10.11 Hz, 1H), 7.73 (dd, J = 6.61, 12.55 Hz, 1H), 2.80 (m, 1H), 1.20 (m, 2H), 0.81 (m, 2H). ^{19}F NMR (282 MHz, DMSO) δ = −127.25 (dd, J = 9.46, 22.73 Hz, 1F), −145.05 (dd, J = 13.39, 26.56 Hz, 1F). LRMS (ESI) calculated for (M + H^+) 239.20, found 239.23. Retention factor (TLC) = 0.65, 5:1 ethyl acetate: hexanes.

4.3.8. Preparation of 1-cyclopropyl-6,7-difluoro-8-methylquinazoline-2,4(1H,3H)-dione (4b) (UIAA-I-218)

To a stirring solution of **UIAA-I-101** (**3b**) (0.081 g, 0.298 mmol) dissolved in 6.0 mL of distilled THF cooled to −10 °C, KHMDS (1.0 M in THF, 0.65 mL, 0.650 mmol) was added, dropwise, under argon atmosphere. The resulting mixture was then allowed to gradually warm to room temperature over 10 min. A catalytic amount of 18-crown-6 (0.0175 g, 0.066 mmol) was added in one portion to the room temperature mixture. The reaction mixture was then heated to reflux at 105 °C for 2 h. After cooling hot reaction mixture to warm temperature, it was diluted with 10 mL of ethyl acetate and washed/quenched with 20 mL 1 N HCl, 20 mL of distilled water, and finally with 20 mL of saturated brine solution. All of the organic layers were pooled, filtered through Na_2SO_4, and concentrated by rotary evaporation. The residue was triturated with a 1:1 ethyl ether: hexanes solution and then filtered to afford crude **UIAA-I-091 (4b)**, the filtrate was reprocessed to afford a 2nd crop. Both crops were purified via silica gel flash chromatography utilizing a 5:1 ethyl acetate: hexanes isocratic elution to afford pure **UIAA-I-091 (4b)**. 91% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.66 (bs, 1H), 7.81 (t, J = 8.93 Hz, 1H), 3.37 (ddd, J = 3.80, 7.16, 10.56 Hz, 1H), 2.61 (d, J = 3.27 Hz, 3H), 0.88 (q, J = 6.61 Hz, 2H), 0.73 (m, 2H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −125.33 (m, 1F), −140.51 (dd, J = 9.18, 22.21 Hz, 1F). HRMS (ESI) calculated for (M + H^+) 253.0783, found 253.0778. Retention factor (TLC) = 0.60, 5:1 ethyl acetate: hexanes.

4.3.9. Preparation of 1-cyclopropyl-6,7-difluoro-8-methoxyquinazoline-2,4(1H,3H)-dione (4c) (UIAA-I-089)

To a stirring solution of **UIAA-I-077 (3c)** (0.510 g, 1.769 mmol) dissolved in 35 mL of distilled THF cooled to −10 °C, KHMDS (1.0 M in THF, 3.75 mL, 3.750 mmol) was added, dropwise, under argon atmosphere. The resulting mixture was then warmed to room temperature over 10 min. A catalytic amount of 18-crown-6 (0.0175 g, 0.066 mmol) was added in one portion to the room temperature mixture. The reaction mixture was then heated to reflux for 1.5 h. After cooling the reaction mixture to room temperature, it was diluted with 20 mL of ethyl acetate and the organic layer washed/quenched with 80 mL 1 N HCl, 80 mL of distilled water, and finally with 80 mL of saturated brine solution. The organic layer was filtered through Na_2SO_4 and concentrated by rotary evaporation. The residue was triturated with a 1:1 ethyl ether:hexanes solution and then filtered to afford pure **UIAA-I-091 (4c)**, the filtrate was reprocessed to afford a 2nd crop. 78% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.43 (bs, 1H), 7.71 (m, 1H), 4.03 (d, J = 1.97 Hz, 3H), 3.34 (m, 1H), 1.19 (q, J = 6.70 Hz, 2H), 0.73 (m, 2H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −138.39 (dd, J = 9.25, 21.08 Hz, 1F), −140.82 (m, 1F). HRMS (ESI) calculated for (M + H^+) 269.0732, found 269.0722. Retention factor (TLC) = 0.66, 5:1 ethyl acetate: hexanes.

4.4. Scheme 2. Synthesis of N-3-ethylamine Quinazoline-2,4-diones, **7a-c**

4.4.1. Preparation of tert-butyl (2-(1-cycpropyl-6,7-difluoro-2,4-dioxo-1,2-dihydroquinazolin-3(4H)-yl)ethyl)carbamate (5a) (UIAA-I-127)

To a stirring solution of **UIAA-I-091** (**4a**) (0.101 g, 0.424 mmol) and K_2CO_3 (0.145 g, 0.878 mmol) in 5 mL of anhydrous DMF, *N*-Boc-2-bromoethylamine (0.173 g, 0.772 mmol), dissolved in 5 mL of anhydrous DMF, was added dropwise over 5 min at room temperature and under argon atmosphere. The reaction mixture was then allowed to stir at room temperature for 26 h before concentrating by rotary evaporation. The residue was then reconstituted in 15 mL of DCM and washed successively with 30 mL of saturated $NaHCO_3$, 30 mL of distilled water, and finally 30 mL of saturated brine solution. The organic layers were pooled and concentrated by rotary evaporation. The crude oil was then purified by silica gel flash chromatography utilizing a gradient elution from 1:10 to 2:1 ethyl acetate:hexanes to afford pure **UIAA-I-127** (**5a**). 75% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.95 (m, 1H), 7.46 (dd, J = 6.40, 11.64 Hz, 1H), 4.96 (bs, 1H), 4.19 (m, 2H), 3.45 (q, J = 5.66 Hz, 2H), 2.86 (ddd, J = 3.91, 6.97, 10.64 Hz), 1.28 (s, 9H), 0.94 (m, 2H), 0.84 (m, 2H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −124.61 (m, 1F), −142.85 (ddd, J = 6.70, 9.32, 22.23 Hz, 1F). HRMS (ESI) calculated for (M + Na^+) 404.1392, found 404.1382. Retention time (analytical HPLC) = 20.2 min.

4.4.2. Preparation of tert-butyl (2-(1-cycpropyl-6,7-difluoro-8-methyl-2,4-dioxo-1,2-dihydroquinazolin-3(4H)-yl)ethyl)carbamate (5b) (UIAA-I-222)

To a stirring solution of **UIAA-I-218** (**4b**) (0.022 g, 0.087 mmol) and K_2CO_3 (0.028 g, 0.169 mmol) in 1.25 mL of anhydrous DMF, *N*-Boc-2-bromoethylamine (0.034 g, 0.152 mmol), dissolved in 1.25 mL of anhydrous DMF, was added dropwise over 5 min at room temperature and under argon atmosphere. The reaction mixture was then allowed to stir at room temperature for 18 h before concentrating by rotary evaporation. The residue was then reconstituted in 10 mL of DCM and washed successively with 20 mL of saturated $NaHCO_3$, 20 mL of distilled water, and finally 20 mL of saturated brine solution. The organic layers were pooled, filtered through Na_2SO_4 and concentrated by rotary evaporation to afford pure **UIAA-I-222** (**5b**). Quantitative yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.80 (t, J = 9.07 Hz, 1H), 4.95 (bs, 1H), 4.18 (m, 2H), 3.49 (q, J = 5.45 Hz, 2H), 3.40 (m, 1H), 3.16 (d, 3H), 2.34 (m, 2H), 2.10 (dd, J = 12.89, 16.05 Hz, 2H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −126.57 (d, J = 16.68 Hz, 1F), −141.30 (dd, J = 9.32, 22.30 Hz, 1F). HRMS (ESI) calculated for (M + Na^+) 418.1549, found 418.1555. Retention time (analytical HPLC) = 20.9 min.

4.4.3. Preparation of tert-butyl (2-(1-cycpropyl-6,7-difluoro-8-methoxy-2,4-dioxo-1,2-dihydroquinazolin-3(4H)-yl)ethyl)carbamate (5c) (UIAA-I-117)

To a stirring solution of **UIAA-I-089** (**4c**) (0.100 g, 0.373 mmol) and K_2CO_3 (0.140 g, 0.847 mmol) in 5 mL of anhydrous DMF, *N*-Boc-2-bromoethylamine (0.150 g, 0.669 mmol), dissolved in 5 mL of anhydrous DMF, was added dropwise over 5 min at room temperature and under argon atmosphere. The reaction mixture was then allowed to stir at room temperature for 22 h before concentrating by rotary evaporation. The residue was then reconstituted in 10 mL of DCM and washed successively with 20 mL of saturated $NaHCO_3$, 20 mL of distilled water, and finally 20 mL of saturated brine solution. The organic layers were pooled, filtered through Na_2SO_4 and concentrated by rotary evaporation. The crude oil was then purified by silica gel flash chromatography utilizing a gradient elution from 1:10 to 2:1 ethyl acetate:hexanes to afford pure **UIAA-I-117** (**5c**). Quantitative yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.72 (dd, J = 8.21, 9.45 Hz, 1H), 4.89 (bs, 1H), 4.19 (m, 2H), 4.01 (d, J = 1.89 Hz, 3H), 3.49 (d, J = 4.68, 2H) 3.38 (m, 1H), 1.32 (s, 9H), 1.17 (m, 2H), 0.74 (s, 2H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −139.20 (s, 1F), −141.96 (s, 1F). HRMS (ESI) calculated for (M + Na^+) 434.1498, found 434.1490. Retention time (analytical HPLC) = 21.0 min.

4.4.4. Preparation of (S)-tert-butyl ((1-(3-(2-aminoethyl)-1-cyclopropyl-6-fluoro-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-7-yl)pyrrolidin-3-yl)methyl) carbamate (6a) (UIAA-I-129)

To a stirring solution of **UIAA-I-127** (**5a**) (0.115 g, 0.302 mmol) and (*R*)-*tert*-butyl (pyrrolidin-3-ylmethyl)carbamate (3.0 M in ACN, 0.295 mL, 0.885 mmol) in 20 mL of distilled acetonitrile, triethylamine (0.150 mL, 1.067 mmol) was added, dropwise, at room temperature and under argon atmosphere. The reaction mixture was then heated to reflux at 95 °C for 30 h. The reaction was then cooled to room temperature and concentrated by rotary evaporation. The resulting residue was then resuspended in 10 mL of DCM and successively washed with 30 mL of distilled water and 30 mL of saturated brine solution. The residue was then purified via silica gel flash chromatography utilizing a gradient elution from 1:6 to 3:1 ethyl acetate:hexanes to afford pure **UIAA-I-129** (**6a**). Quantitative yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.66 (d, J = 13.89 Hz, 1H), 6.62 (d, J = 7.18 Hz, 1H), 5.09 (bs, 1H), 4.77 (bs, 1H), 4.20 (m, 2H), 3.67 (m, 2H), 3.60 (m, 1H), 3.45 (d, J = 4.39 Hz, 2H), 3.33 (m, 1H), 3.25 (m, 2H), 2.82 (m, 1H), 2.14 (m, 1H), 1.80 (dd, J = 8.17, 12.57 Hz, 2H), 1.47 (s, 9H), 1.36 (s, 9H), 1.27 (m, 2H), 0.94 (m, 2H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −134.14 (s, 1F). HRMS (ESI) calculated for (M + Na^+) 584.2860, found 584.2866. Retention time (analytical HPLC) = 21.8 min.

4.4.5. Preparation of (S)-tert-butyl ((1-(3-(2-aminoethyl)-1-cyclopropyl-6-fluoro-8-methyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-7-yl)pyrrolidin-3-yl)methyl)carbamate (6b) (UIAA-I-224)

To a stirring solution of **UIAA-I-222** (**5b**) (0.035 g, 0.0885 mmol) and (*R*)-*tert*-butyl (pyrrolidin-3-ylmethyl)carbamate (0.050 g, 0.250 mmol) in 10 mL of distilled acetonitrile, triethylamine (0.040 mL, 0.285 mmol) was added, dropwise, at room temperature and under argon atmosphere. The reaction mixture was then heated to reflux at 95 °C for 7 days. The reaction was then cooled to room temperature and concentrated by rotary evaporation. The resulting residue was then resuspended in 10 mL of DCM and successively washed with 30 mL of saturated $NaHCO_3$, 30 mL of distilled water and 30 mL of saturated brine solution. The residue was then purified via silica gel flash chromatography utilizing a gradient elution from 1:6 to 3:1 ethyl acetate:hexanes to afford pure **UIAA-I-224** (**6b**). 25% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.57 (J = 12.90 Hz, 1H), 5.05 (bs, 1H), 4.74 (bs, 1H), 4.18 (m, 2H), 3.49 (m, 2H), 3.39 (m, 2H), 3.25 (m, 2H), 2.51 (m, 1H), 2.41 (s, 3H), 2.10 (m, 1H), 1.73 (dt, J = 7.74, 19.88 Hz, 2H), 1.46 (s, 9H), 1.30 (d, J = 22.70 Hz, 9H), 1.13 (t, J = 6.70 Hz, 2H), 0.86 (m, 2H), 0.65 (m, 2H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −126.69 (d, J = 12.71 Hz, 1F). HRMS (ESI) calculated for (M + Na^+) 598.3017, found 598.3019. Retention time (analytical HPLC) = 22.3 min.

4.4.6. Preparation of (S)-tert-butyl ((1-(3-(2-aminoethyl)-1-cyclopropyl-6-fluoro-8-methoxy-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-7-yl)pyrrolidin-3-yl)methyl)carbamate (6c) (UIAA-I-125)

To a stirring solution of **UIAA-I-117** (**5b**) (0.153 g, 0.372 mmol) and (*R*)-*tert*-butyl (pyrrolidin-3-ylmethyl)carbamate (3.0 M in ACN, 0.350 mL, 1.05 mmol) in 2 mL of distilled acetonitrile, triethylamine (0.170 mL, 1.209 mmol) was added, dropwise, at room temperature and under argon atmosphere. The reaction mixture was then heated to reflux at 95 °C for 26 h. The reaction was then cooled to room temperature and concentrated by rotary evaporation. The resulting residue was then resuspended in 10 mL of DCM and successively washed with 30 mL of distilled water and 30 mL of saturated brine solution. The residue was then purified via silica gel flash chromatography utilizing a gradient elution from 1:6 to 1:0 ethyl acetate:hexanes to afford pure **UIAA-I-125** (**6b**). Quantitative yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.49 (d, J = 13.59 Hz, 1H), 5.05 (bs, 1H), 4.72 (bs, 1H), 4.18 (d, J = 3.67 Hz, 2H), 3.68 (m, 3H), 3.52 (s, 3H), 3.44 (m, 3H), 3.29 (m, 3H), 2.46 (m, 1H), 2.11 (m, 1H), 2.06 (s, 1H), 1.47 (s, 9H), 1.35 (s, 9H), 0.87 (m, 2H), 0.66 (m, 2H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −127.15 (s, 1F). HRMS (ESI) calculated for (M + H^+) 614.30, found 614.33.

Retention time (analytical HPLC) = 22.3 min.

4.4.7. Preparation of (S)-3-(2-aminoethyl)-7-(3-(aminomethyl)pyrrolidin-1-yl)-1-cyclopropyl-6-fluoroquinazoline-2,4-(1H,3H)-dione (7a) (UIAA-I-133)

UIAA-I-129 (**6a**) (0.1826 g, 0.325 mmol) was dissolved in 30 mL of solution containing a mixture of 1:1 acetonitrile and aqueous 3 N HCl. The resulting reaction mixture was stirred in ambient conditions for 22 h. The acetonitrile and HCl was removed by rotary evaporation until only an aqueous layer remained in the reaction flask. The aqueous layer was subject to lyophilizing conditions to afford pure **UIAA-I-133** (**7a**) as the hydrochloride salt. Quantitative yield. ^{1}H NMR (300 MHz, DMSO) δ = 8.41 (bs, 2H), 8.04 (bs, 2H), 7.50 (d, J = 13.85 Hz, 1H), 6.73 (d, J = 7.58 Hz, 1H), 4.11 (t, J = 4.58 Hz, 2H), 3.71 (dd, J = 6.82, 16.58 Hz, 3H), 3.39 (m, 1H), 3.02 (d, J = 4.95 Hz, 2H), 2.94 (s, 2H), 2.80 (s, 1H), 2.64 (m, 1H), 2.16 (m, 1H), 1.83 (m, 1H), 1.27 (d, J = 5.76 Hz, 2H), 0.81 (d, J = 5.93 Hz, 2H). ^{19}F NMR (282 MHz, DMSO) δ = −100.00 (s, 1F). HRMS (ESI) calculated for (M + Na^+) 384.1812, found 384.1795. Retention time (analytical HPLC) = 9.9 min.

4.4.8. Preparation of (S)-3-(2-aminoethyl)-7-(3-(aminomethyl)pyrrolidin-1-yl)-1-cyclopropyl-6-fluoro-8-methyl-quinazoline-2,4-(1H,3H)-dione (7b) (UIAA-I-227)

UIAA-I-224 (**6b**) (0.0127 g, 0.0221 mmol) was dissolved in 10 mL of solution containing a mixture of 1:1 acetonitrile and aqueous 3 N HCl. The resulting reaction mixture was stirred in ambient conditions for 22 h. The acetonitrile and HCl was removed by rotary evaporation until only an aqueous layer remained in the reaction flask. The aqueous layer was subject to lyophilizing conditions to afford pure **UIAA-I-227** (**7b**) as the hydrochloride salt. 77% yield. ^{1}H NMR (300 MHz, DMSO) δ = 8.19 (bs, 2H), 7.93 (bs, 2H), 7.44 (d, J = 13.12 Hz, 1H), 4.09 (s, 2H), 3.51 (dd, J = 8.14, 17.29 Hz, 3H), 3.06 (m, 1H),2.98 (m, 2H), 2.75 (m, 1H), 2.62 (m, 3H), 2.13 (m, 1H), 1.76 (dd, J = 7.95, 11.48 Hz, 1H), 1.23 (s, 1H), 1.05 (d, J = 6.56 Hz, 2H), 0.63 (m, 2H). ^{19}F NMR (282 MHz, DMSO) δ = −127.97 (d, J = 13.36, 1F). HRMS (ESI) calculated for (M + H^+) 376.2143, found 376.2146. Retention time (analytical HPLC) = 10.3 min.

4.4.9. Preparation of (S)-3-(2-aminoethyl)-7-(3-(aminomethyl)pyrrolidin-1-yl)-1-cyclopropyl-6-fluoro-8-methoxy-quinazoline-2,4-(1H,3H)-dione (7c) (UIAA-I-131)

UIAA-I-125 (**6c**) (0.215 g, 0.363 mmol) was dissolved in 30 mL of solution containing a mixture of 1:1 acetonitrile and aqueous 3 N HCl. The resulting reaction mixture was allowed to stir in ambient conditions for 23 h. The acetonitrile and HCl was removed by rotary evaporation until only an aqueous layer remained in the reaction flask. The aqueous layer was subject to lyophilizing conditions to afford pure **UIAA-I-131** (**7c**) as the hydrochloride salt. 97% yield. ^{1}H NMR (300 MHz, DMSO) δ = 8.36 (bs, 2H), 8.10 (bs, 2H), 7.35 (, J = 13.54 Hz, 1H), 4.08 (t, J = 5.51 Hz, 2H), 3.68 (m, 1H), 3.57 (d, J = 12.15 Hz, 2H), 3.49 (s, 3H), 3.43 (d, J = 6.55 Hz, 1H), 3.22 (dd, J = 5.20, 8.77 Hz, 1H), 3.03 (d, J = 5.08 Hz, 2H), 2.91 (s, 2H), 2.57 (dd, J = 6.81, 13.55 Hz, 1H), 2.12 (dd, J = 6.25, 12.04 Hz, 1H), 1.76 (m, 1H), 0.92 (dd, J = 10.75, 13.81 Hz, 2H), 0.67 dd, J = 7.03, 23.04 Hz, 2H). ^{19}F NMR (282 MHz, DMSO) δ = −100.00 (s, 1F). HRMS (ESI) calculated for (M + Na^+) 414.1917, found 414.1911. Retention time (analytical HPLC) = 10.2 min.

4.5. Scheme 3. Synthesis of N-3-ethyl guanidine Quinazoline-2,4-diones, ***13a-c***

4.5.1. Preparation of N-(2-cyclopropyl-6,7-difluoro-2,4-dioxo-1,2-dihydroquinazolin-3(4H)-yl)ethyl)-2,2,2-trifluoroacetamide (9a) (UIAA-II-063)

UIAA-II-057 (5a) (0.186 g, 0.487 mmol) was dissolved in 20 mL of a 1:1 DCM:TFA solution at room temperature. The resulting reaction mixture was stirred in ambient conditions for 19 h. The reaction mixture was then concentrated via rotary evaporation. The resulting crude oil was resuspended in 10 mL of DCM and washed several times with 10 mL of saturated $NaHCO_3$ solution. The aqueous layer was back extracted > 5X to make sure all product was collected. The organic layers were then pooled and concentrated to afford **UIAA-II-061** (**8a**), which was taken to the next step without further purification (Crude yield = 132.9 mg; 97%). **UIAA-II-061** (**8a**) (0.133 g, 0.473 mmol) and triethylamine (0.100 mL, 0.719 mmol) was dissolved in 15 mL of anhydrous chloroform and cooled to 0 °C under argon atmosphere. 2,2,2-trifluoroacetic anhydride (0.030 mL, 0.216 mmol) was added to the cool solution, dropwise and the reaction mixture was then allowed to warm to room temperature and consequently stir at room temperature for 48 h. The reaction mixture was concentrated by rotary evaporation and purified via silica gel flash chromatography utilizing a gradient elution from 1:4 to 1:1 ethyl acetate:hexanes to afford pure **UIAA-II-063** (**9a**). 54% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.98 (dd, J = 8.55, 9.51 Hz, 1H), 7.52 (dd, J = 6.35, 11.52 Hz, 1H), 7.44 (bs, 1H), 4.35 (m, 2H), 3.70 (dd, J = 5.00, 10.22 Hz, 2H), 2.88 (m, 1H), 1.35 (m, 2H), 0.85 (m, 2H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −76.08 (s, 3F), −123.30 (m, 1F), −141.75 (ddd, J = 6.38, 9.50, 22.24 Hz, 1F). HRMS (ESI) calculated for (M + Na^+) 400.0697, found 400.0689. Retention time (analytical HPLC) = 20.1 min.

4.5.2. Preparation of N-(2-cyclopropyl-6,7-difluoro-8-methyl-2,4-dioxo-1,2-dihydroquinazolin-3(4H)-yl)ethyl)-2,2,2-trifluoroacetamide (9b) (UIAA-II-065)

UIAA-II-056 (5b) (0.173 g, 0.434 mmol) was dissolved in 20 mL of a 1:1 DCM:TFA solution at room temperature. The resulting reaction mixture was allowed to stir in ambient conditions for 19 h. The reaction mixture was then concentrated via rotary evaporation. The resulting crude oil was resuspended in 10 mL of DCM and washed several times with 10 mL of saturated $NaHCO_3$ solution. The aqueous layer was back extracted > 5X to make sure all product was collected. The organic layers were then pooled and concentrated to afford **UIAA-II-062** (**8b**), which was taken to the next step without further purification (Crude yield = 115.8 mg; 86%).**UIAA-II-062** (**8b**) (0.115 g, 0.377 mmol) and triethylamine (0.135 mL, 0.960 mmol) was dissolved in 15 mL of anhydrous chloroform and cooled to 0 °C under argon atmosphere. 2,2,2-trifluoroacetic anhydride (0.080 mL, 0.576 mmol) was added to the cool solution, dropwise and the reaction mixture was then allowed to warm to room temperature and consequently stir at room temperature for 16 h. The reaction mixture was concentrated by rotary evaporation and purified via silica gel flash chromatography utilizing a gradient elution from 1:4 to 1:1 ethyl acetate:hexanes to afford pure **UIAA-II-065** (**9b**). 90% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.81 (t, J = 8.90 Hz, 1H), 7.50 (bs, 1H), 4.31 (dt, J = 4.33, 10.70 Hz, 2H), 3.72 (dd, J = 4.97, 10.12 Hz, 2H), 3.41 (m, 1H), 2.61 (d, J = 3.19 Hz, 3H), 0.87 (m, 2H), 0.71 (m, 2H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −76.03 (s, 3F), −125.25 (ddd, J = 3.27, 8.40, 22.20 Hz, 1F), −140.24 (dd, J = 9.2, 22.29). HRMS (ESI) calculated for (M + Na^+) 414.0853, found 414.0855. Retention time (analytical HPLC) = 20.6 min.

4.5.3. Preparation of N-(2-cyclopropyl-6,7-difluoro-8-methoxy-2,4-dioxo-1,2-dihydroquinazolin-3(4H)-yl)ethyl)-2,2,2-trifluoroacetamide (9c) (UIAA-II-069)

UIAA-II-064 (5c) (0.269 g, 0.653 mmol) was dissolved in 20 mL of a 1:1 DCM:TFA solution at room temperature. The resulting reaction mixture was allowed to stir in ambient conditions for 14 h. The reaction mixture was then concentrated via rotary evaporation. The resulting crude oil was resuspended in 10 mL of DCM and washed several times with 10 mL of saturated $NaHCO_3$ solution. The aqueous layer was back extracted > 5X to make sure all product was collected. The organic layers were then pooled and concentrated to afford **UIAA-II-068** (**8c**), which was taken to the next step without further purification (Crude

yield = 206.6 mg; 98%).**UIAA-II-068** (**8c**) (0.207 g, 0.639 mmol) and triethylamine (0.230 mL, 1.636 mmol) was dissolved in 10 mL of anhydrous chloroform and cooled to 0 °C under argon atmosphere. 2,2,2-trifluoroacetic anhydride (0.130 mL, 0.935 mmol) was added to the cool solution, dropwise and the reaction mixture was then allowed to warm to room temperature and consequently stir at room temperature for 16 h. The reaction mixture was concentrated by rotary evaporation and purified via silica gel flash chromatography utilizing a gradient elution from 1:4 to 1:1 ethyl acetate:hexanes to afford pure **UIAA-II-069** (**9c**). 73% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.69 (t, J = 8.69 Hz, 1H), 7.45 (bs, 1H), 4.32 (m, 2H), 4.03 (d, J = 1.72 Hz, 3H), 3.70 (d, J = 4.87 Hz, 2H), 3.39 (dd, 3.37, 6.88 Hz, 1H), 1.20 (q, J = 6.68 Hz, 2H), 0.70 (d, J = 3.21, 2H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −76.03 (s, 3F), −138.16 (dd, J = 9.29, 21.10 Hz, 1F), −140.84 (dd, J = 6.24, 21.25 Hz, 1F). HRMS (ESI) calculated for (M + Na^+) 430.0802, found 430.0797. Retention time (analytical HPLC) = 20.8 min.

4.5.4. Preparation of (S)-tert-butyl ((1-(1-cyclopropyl-6-fluoro-2,4-dioxo-3-(2-(2,2,2-trifluoroacetamido)ethyl)-1,2,3,4-tetrahydroquinazolin-7-yl) pyrrolidin-3-yl)methyl)carbamate (10a) (UIAA-II-066)

To a stirring solution of **UIAA-II-063** (**9a**) (0.080 g, 0.212 mmol) and (*R*)-*tert*-butyl (pyrrolidin-3-ylmethyl)carbamate (0.111 g, 0.554 mmol) in 1.0 mL of anhydrous DMSO, triethylamine (0.090 mL, 0.640 mmol) was added, dropwise, at room temperature and under argon atmosphere. The reaction mixture was then heated to 55 °C for 16 h. The product was then precipitated out with cold nanopure water and filtered. The crude product was then purified via silica gel flash chromatography utilizing an isocratic elution consisting of only ethyl acetate to afford pure **UIAA-II-066** (**10a**). 91% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.83 (bs, 1H), 7.65 (d, J = 13.75 Hz, 1H), 6.64 (d, J = 7.31 Hz, 1H), 4.78 (bs, 1H), 4.34 (dd, J = 3.82, 6.27 Hz, 2H), 3.67 (m, 4H), 3.36 (t, J = 7.21 Hz, 1H), 3.26 (m, 2H), 2.83 (m, 1H), 2.55 (m, 1H), 2.16 (dd, J = 5.16, 11.56 Hz, 1H), 1.81 (dt, J = 7.58, 19.77 Hz, 1H), 1.71 (s, 9H), 1.29 (m, 2H), 0.92 (m, 2H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −76.02 (s, 3F), −133.54 (s, 1F). HRMS (ESI) calculated for (M + Na^+) 580.2159, found 580.2228. Retention time (analytical HPLC) = 21.8 min.

4.5.5. Preparation of (S)-tert-butyl ((1-(1-cyclopropyl-6-fluoro-8-methoxy-2,4-dioxo-3-(2-(2,2,2-trifluoroacetamido)ethyl)-1,2,3,4-tetrahydroquinazolin-7-yl)pyrrolidin-3-yl)methyl)carbamate (10c) (UIAA-II-072)

To a stirring solution of **UIAA-II-069** (**9c**) (0.190 g, 0.466 mmol) and (*R*)-*tert*-butyl (pyrrolidin-3-ylmethyl)carbamate (0.232 g, 1.158 mmol) in 2.0 mL of anhydrous DMSO, triethylamine (0.197 mL, 1.401 mmol) was added, dropwise, at room temperature and under argon atmosphere. The reaction mixture was then heated to 55 °C for 4 days. The product was then precipitated out with cold nanopure water and filtered. The crude product was then purified via silica gel flash chromatography utilizing an isocratic elution consisting of only ethyl acetate to afford pure **UIAA-II-072** (**10c**). Quantitative yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.78 (bs, 1H), 7.49 (d, J = 13.53 Hz, 1H), 4.72 (bs, 1H), 4.32 (d, J = 3.82 Hz, 2H), 3.66 (m, 3H), 3.51 (d, J = 7.05 Hz, 3H), 3.46 (s, 1H), 3.33 (m, 2H), 3.26 (m, 2H), 2.47 (m, 1H), 2.11 (dd, J = 8.09, 14.10 Hz), 1.71 (dd, 8.12, 12.04 Hz, 2H), 1.47 (s, 9H), 0.90 (m, 2H), 0.63 (m, 2H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −76.01 (s, 3F), −126.41 (d, J = 12.14 Hz, 1F). HRMS (ESI) calculated for (M + H^+) 588.2440, found 588.2861. Retention time (analytical HPLC) = 22.2 min.

4.5.6. Preparation of (S)-tert-butyl ((1-(3-(2-aminoethyl)-1-cyclopropyl-6-fluoro-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-7-yl)pyrrolidin-3-yl)methyl) carbamate (11a) (UIAA-II-070)

UIAA-II-066 (**10a**) (0.108 g, 0.194 mmol) was dissolved in 6 mL of a 6 N NH_4OH MeOH solution at room temperature. The resulting reaction mixture was allowed to stir at room temperature for 14 h. The reaction mixture was then concentrated by rotary evaporation. The residue was then reconstituted in 10 mL of DCM and washed with saturated $NaHCO_3$ and the aqueous layer was back extracted with DCM 5X. All organic layers were pooled, filtered through Na_2SO_4, and concentrated by rotary evaporation. Purified crude product by silica gel flash chromatography utilizing a gradient elution from 100% ethyl acetate to 100% DCM, and finally to 10% MeOH in DCM (all eluents contained 2–3% triethylamine) to afford pure **UIAA-II-070** (**11a**). 84% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.66 (d, J = 13.82 Hz, 1H), 6.63 (d, J = 7.38 Hz, 1H), 4.79 (bs, 1H), 4.12 (bs, 2H), 3.68 (m, 2H), 3.59 (m, 2H), 3.32 (m, 2H), 3.26 (m, 2H), 2.81 (m, 1H), 2.54 (m, 1H), 2.15 (dd, J = 6.78, 13.17 Hz, 1H), 1.82 (dd, J = 6.44, 14.34 Hz, 1H), 1.47 (s, 9H), 1.28 (s, 2H), 0.91 (m, 2H), 0.86 (m, 2H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −134.14 (s, 1F). HRMS (ESI) calculated for (M + H^+) 462.2511, found 462.2535. Retention time (analytical HPLC) = 17.4 min.

4.5.7. Preparation of (S)-tert-butyl ((1-(3-(2-aminoethyl)-1-cyclopropyl-6-fluoro-8-methoxy-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-7-yl)pyrrolidin-3-yl)methyl)carbamate (11c) (UIAA-II-074)

UIAA-II-072 (**10c**) (0.284 g, 0.483 mmol) was dissolved in 15 mL of a 6 N NH_4OH MeOH solution at room temperature. The resulting reaction mixture was allowed to stir at room temperature for 14 h. The reaction mixture was then concentrated by rotary evaporation. The residue was then reconstituted in 10 mL of DCM and washed with saturated $NaHCO_3$ and the aqueous layer was back extracted with DCM 5X. All organic layers were pooled, filtered through Na_2SO_4, and concentrated by rotary evaporation. Purified crude product by silica gel flash chromatography utilizing a gradient elution from 100% ethyl acetate to 100% DCM, and finally to 10% MeOH in DCM (all eluents contained 2–3% triethylamine) to afford pure **UIAA-II-074** (**11c**). 98% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.47 (d, J = 13.55 Hz, 1H), 4.74 (bs, 1H), 4.13 (bs, 2H), 3.66 (m, 3H), 3.51 (s, 3H), 3.45 (m, 1H), 3.33 (m, 2H), 3.27 (dd, J = 6.74, 13.08 Hz, 2H), 3.03 (m, 2H), 2.46 (m, 2H), 2.10 (dd, J = 7.97, 14.26 Hz, 2H), 1.70 (dd, J = 7.97, 11.87 Hz, 2H), 1.47 (s, 9H), 1.08 (m, 2H), 0.62 (m, 2H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −127.04 (s, 1F). HRMS (ESI) calculated for (M + H^+) 492.2617, found 492.2594. Retention time (analytical HPLC) = 18.5 min.

4.5.8. Preparation of (S)-tert-butyl ((1-(1-cyclopropyl-6-fluoro-3-(2-bis-(tert-butylguanidine-carbamate)ethyl)-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-7-yl)pyrrolidin-3-yl)methyl)carbamate (12a) (UIAA-II-077)

UIAA-II-070 (**11a**) (0.0745 g, 0.161 mmol) and N,N'-bis-Boc-guanylpyrazole (0.0767 g, 0.247 mmol) was dissolved in 3 mL of chloroform (anhydrous) at room temperature and under argon atmosphere. The reaction mixture was then heated to 40 °C for 72 h. The reaction mixture was then cooled to room temperature and concentrated by rotary evaporation. The resulting residue was then purified via flash chromatography, utilizing a gradient elution from 0 to 50% ethyl acetate in cyclohexane to afford pure **UIAA-II-077** (**12a**). 52% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 11.44 (bs, 1H), 8.49 (bs, 1H), 7.65 (d, J = 13.63 Hz, 1H), 6.62 (d, J = 7.39 Hz, 1H), 4.8 (bs, 1H), 4.28 (dd, J = 9.41, 15.19 Hz, 2H), 3.74 (dd, J = 5.48, 10.81 Hz, 2H), 3.58 (d, J = 7.48 Hz, 2H), 3.32 (dd, J = 6.97, 14.55 Hz, 2H), 3.24 (m, 2H), 2.79 (m, 1H), 2.53 (m, 1H), 2.14 (dd, J = 7.87, 12.40 Hz, 1H), 1.48 (s, 9H), 1.46 (s, 18H), 0.95 (m, 2H), 0.86 (m, 2H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −134.31 (s, 1F). HRMS (ESI) calculated for (M + H^+) 704.3778, found 704.3807. Retention time (analytical HPLC) = 29.6 min.

4.5.9. Preparation of (S)-tert-butyl ((1-(1-cyclopropyl-6-fluoro-3-(2-bis-(tert-butylguanidine-carbamate)ethyl)-8-methyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-7-yl)pyrrolidin-3-yl)methyl)carbamate (12b) (UIAA-II-081)

To a stirring solution of **UIAA-II-065** (**9b**) (0.118 g, 0.301 mmol)

and (*R*)-*tert*-butyl (pyrrolidin-3-ylmethyl)carbamate (1.5 M in DMSO, 0.400 mL, 0.600 mmol) in 1.0 mL of anhydrous DMSO, triethylamine (0.127 mL, 0.903 mmol) was added, dropwise, at room temperature and under argon atmosphere. The reaction mixture was then heated to 55 °C for 4 days. The product was then precipitated out with cold nanopure water and filtered. The crude product, **UIAA-II-073** (**10b**), was taken to the next step without further purification (Crude yield = 151.9 mg, 89%). **UIAA-II-073** (**10b**) (0.1519 g, 0.2657 mmol) was dissolved in 20 mL of a 6 N NH_4OH MeOH solution at room temperature. The resulting reaction mixture was allowed to stir at room temperature for 14 h. The reaction mixture was then concentrated by rotary evaporation. The residue was then reconstituted in 10 mL of DCM and washed with saturated $NaHCO_3$ and the aqueous layer was back extracted with DCM 5X. All organic layers were pooled, filtered through Na_2SO_4, and concentrated by rotary evaporation. The crude product, **UIAA-II-078** (**11b**), was taken to the next step without further purification (Crude yield = 121.2 mg, 96% yield. **UIAA-II-078** (**11b**) (0.121 g, 0.254 mmol) and N,N'-bis-Boc-guanylpyrazole (0.121 g, 0.390 mmol) were dissolved in 3 mL of chloroform (anhydrous) at room temperature and under argon atmosphere. The reaction mixture was then heated to 40 °C for 72 h. The reaction mixture was then cooled to room temperature and concentrated by rotary evaporation. The resulting residue was then purified via flash chromatography, utilizing a gradient elution from 0 to 50% ethyl acetate in cyclohexane to afford pure **UIAA-II-081** (**12b**). 51% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 11.45 (bs, 1H), 8.51 (bs, 1H), 7.61 (dd, *J* = 7.41, 21.20 Hz, 1H), 4.72 (bs, 1H), 4.28 (d, *J* = 2.94 Hz, 2H), 3.78 (m, 2H), 3.50 (m, 3H), 3.37 (m, 1H), 3.28 (m, 2H), 2.49 (d, *J* = 12.38 Hz, 1H), 2.41 (s, 3H), 2.12 (m, 1H), 1.74 (dd, 7.34, 11.98 Hz, 1H), 1.49 (s, 9H), 1.47 (s, 18H), 1.28 (t, *J* = 7.17 Hz, 2H), 1.12 (t, J = 8.12, 2H), 0.61 (m, 2H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −126.83 (s, 1F). HRMS (ESI) calculated for (M + H^+) 718.3934, found 718.4000. Retention time (analytical HPLC) = 29.4 min.

4.5.10. Preparation of (S)-tert-butyl ((1-(1-cyclopropyl-6-fluoro-3-(2-bis-(tert-butylguanidine-carbamate)ethyl)-8-methoxy-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-7-yl)pyrrolidin-3-yl)methyl)carbamate (12c) (UIAA-II-076)

UIAA-II-074 (**11c**) (0.233 g, 0.474 mmol) and N,N'-bis-Boc-guanylpyrazole (0.223 g, 0.719 mmol) was dissolved in 3 mL of chloroform (anhydrous) at room temperature and under argon atmosphere. The reaction mixture was then heated to 40 °C for 72 h. The reaction mixture was then cooled to room temperature and concentrated by rotary evaporation. The resulting residue was then purified via flash chromatography, utilizing a gradient elution from 0 to 50% ethyl acetate in cyclohexane to afford pure **UIAA-II-076** (**12c**). 80% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 11.44 (bs, 1H), 8.49 (t, *J* = 5.06 Hz, 1H), 7.46 (d, *J* = 13.54 Hz, 1H), 4.82 (bs, 1H), 4.23 (dd, 7.52, 13.16 Hz, 2H), 3.74 (dd, *J* = 5.40, 10.88 Hz, 2H), 3.64 (m, 2H), 3.47 (d, *J* = 8.82 Hz, 3H), 3.42 (m, 1H), 3.27 (m, 3H), 2.44 (m, 1H), 2.08 (dd, *J* = 5.00, 10.61 Hz), 1.67 (dd, *J* = 3.97, 7.98 Hz), 1.57 (m, 1H), 1.47 (s, 9H), 1.45 (s, 18H), 1.05 (m, 2H), 0.61 (m, 2H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −127.21 (d, 1F). HRMS (ESI) calculated for (M + H^+) 734.3883, found 734.3892. Retention time (analytical HPLC) = 30.0 min.

4.5.11. Preparation of (S)-1-(2-(7-(3-(aminomethyl)pyrrolidin-1-yl)-1-cyclopropyl-6-fluoro-2,4-dioxo-1,2-dihydroquinazolin-3(4H(-yl)ethyl) guanidine (13a) (UIAA-II-080)

UIAA-II-077 (**12a**) (0.0565 g, 0.0803 mmol) was dissolved in 20 mL of a 1:1 mixture of 4 N HCl (aq.) and acetonitrile. The solution was stirred in ambient conditions for 10 h and consequently concentrated via rotary evaporation to remove all of the acetonitrile. The resulting aqueous solution was frozen and lyophilized to afford pure **UIAA-II-080** (**13a**) as the HCl salt. Quantitative yield. ^{1}H NMR (300 MHz, DMSO) δ = 8.33 (bs, 2H), 7.86 (bs, 1H), 7.51 (d, J = 13.95, 1H), 7.32 (bs, 1H), 7.15 (bs, 1H), 6.73 (d, J = 7.59, 1H), 3.99 (m, 2H), 3.73 (m, 1H), 3.60 (m, 2H), 3.39 (m, 3H), 2.94 (m, 2H), 2.82 (m, 1H), 2.63 (m, 1H), 2.15 (m, 1H), 1.81 (m, 1H), 1.28 (m, 2H), 0.83 (m, 2H). ^{19}F NMR (282 MHz, DMSO) δ = −134.84 (s, 1F). HRMS (ESI) calculated for (M + H^+) 404.2205, found 404.2198. Retention time (analytical HPLC) = 11.2 min.

4.5.12. Preparation of (S)-1-(2-(7-(3-(aminomethyl)pyrrolidin-1-yl)-1-cyclopropyl-6-fluoro-8-methyl-2,4-dioxo-1,2-dihydroquinazolin-3(4H(-yl) ethyl)guanidine (13b) (UIAA-II-082)

UIAA-II-081 (**12b**) (0.0842 g, 0.117 mmol) was dissolved in 20 mL of a 1:1 mixture of 4 N HCl (aq.) and acetonitrile. The solution was stirred in ambient conditions for 10 h and consequently concentrated via rotary evaporation to remove all of the acetonitrile. The resulting aqueous solution was frozen and lyophilized to afford pure **UIAA-II-082** (**13b**) as the HCl salt. Quantitative yield. ^{1}H NMR (300 MHz, DMSO) δ = 8.24 (bs, 2H), 7.82 (d, *J* = 7.82 Hz, 1H), 7.67 (t, *J* = 6.17 Hz, 1H), 7.41 (dd, 1H), 7.28 (bs, 1H), 7.15 (bs, 1H), 3.97 (m, 2H), 3.50 (dd, *J* = 8.98, 17.66 Hz, 2H), 3.45 (m, 2H), 3.38 (dd, *J* = 5.85, 11.87 Hz, 2H), 3.33 (dd, *J* = 6.90, 10.12 Hz, 2H), 2.91 (m, 2H), 2.57 (dd, *J* = 6.76, 13.86 Hz, 1H), 2.34 (d, *J* = 19.03 Hz, 3H), 2.12 (td, *J* = 6.87, 12.04 Hz, 1H), 1.04 (q, *J* = 5.62 Hz, 2H), 0.59 (m, 2H). ^{19}F NMR (282 MHz, DMSO) δ = −127.94 (d, *J* = 12.76 Hz, 1F). HRMS (ESI) calculated for (M + H^+) 418.2361, found 418.2343. Retention time (analytical HPLC) = 11.9 min.

4.5.13. Preparation of (S)-1-(2-(7-(3-(aminomethyl)pyrrolidin-1-yl)-1-cyclopropyl-6-fluoro-8-methoxy-2,4-dioxo-1,2-dihydroquinazolin-3(4H (-yl)ethyl)guanidine (13c) (UIAA-II-079)

UIAA-II-076 (**12c**) (0.273 g, 0.372 mmol) was dissolved in 20 mL of a 1:1 mixture of 4 N HCl (aq.) and acetonitrile. The solution was stirred in ambient conditions for 10 h and consequently concentrated via rotary evaporation to remove all of the acetonitrile. The resulting aqueous solution was frozen and lyophilized to afford pure **UIAA-II-079** (**13c**) as the HCl salt. Quantitative yield. ^{1}H NMR (300 MHz, DMSO) δ = 8.25 (bs, 2H), 7.88 (d, *J* = 2.06 Hz, 1H), 7.73 (bs, 1H), 7.47 (bs, 1H), 7.35 (d, *J* = 13.30 Hz, 1H), 6.44 (bs, 1H), 3.97 (m, 2H), 3.68 (m, 1H), 3.60 (m, 2H), 3.49 (s, 3H), 3.40 (m, 3H), 3.23 (m, 1H), 2.92 (m, 2H), 2.12 (m, 1H), 1.78 (d, *J* = 12.44 Hz, 1H), 0.94 (m, 2H), 0.63 (m, 2H). ^{19}F NMR (282 MHz, DMSO) δ = −127.72 (d, *J* = 13.71 Hz, 1F). HRMS (ESI) calculated for (M + H^+) 434.2310, found 434.2340. Retention time (analytical HPLC) = 11.9 min.

*4.6. Scheme 4. Synthesis of the ethyl 5,6-dihydropyrazolo[1,5-c] quinazoline carboxylates **19a** and **19b***

4.6.1. Preparation of 3-diazo-5,6-difluoroindolin-2-one (15) (UIAA-II-187)

Dissolved 5,6-difluoroindoline-2,3-dione (**14**) (0.714 g, 3.899 mmol) in 25 mL of MeOH, under argon atmosphere, and refluxed the resulting solution at 60 °C for 15 min. p-toluenesulfonyl hydrazide (0.807 g, 4.333 mmol), dissolved in 5 mL of MeOH, was added to the hot solution in one portion. The resulting reaction mix was refluxed, at 60 °C for 24 h. Consequently, the reaction mixture was allowed to cool to room temperature, gradually, and filtered to afford a yellow precipitate. The precipitate was dissolved in 30 mL of distilled water, containing sodium hydroxide (0.331 g, 8.275 mmol), and heated to 64 °C for 4 h. Solid carbon dioxide was added to the cooled reaction mixture to precipitate the pure diazo product, **UIAA-II-187** (**15**), which was filtered and concentrated. 52% yield. ^{1}H NMR (300 MHz, DMSO) δ = 10.83 (bs, 1H), 7.64 (dd, *J* = 7.82, 10.65 Hz, 1H), 6.96 (dd, *J* = 7.07, 10.83 Hz, 1H). ^{19}F NMR (282 MHz, DMSO) δ = −142.37 (m ,1F), −147.72 (ddd, *J* = 6.96, 10.71, 21.72 Hz, 1F). HRMS (ESI) calculated for (M + H^+) 196.0317, found 196.0323. Retention time (analytical HPLC) = 14.9 min.

4.6.2. Preparation of ethyl 8,9-difluoro-5-oxo-5,6-dihydropyrazolo[1,5c] quinazoline-carboxylates (16a-b) (UIAA-II-193a-b)

UIAA-II-187 (**15**) (0.238 g, 1.220 mmol) was dissolved in 6 mL of distilled toluene, ethyl propiolate (0.161 mL, 1.589 mmol) was then added to the solution, in one portion, at room temperature and under argon atmosphere. The resulting reaction mixture was then refluxed, at 115 °C, for 17 h. The cooled reaction mixture was then filtered and washed successively with 30 mL of toluene and 30 mL of hexanes to afford **UIAA-II-193a-b** (**16a-b**), as an off-white amorphous powder. 71% total yield of both **UIAA-II-193a** (**16a**) and **b** (**16b**). **UIAA-II-193a** (**16a**) ^{1}H NMR (300 MHz, DMSO) δ = 12.22 (bs, 1H), 8.39 (dd, *J* = 8.53, 10.78 Hz, 1H), 7.72 (s, 1H), 7.29 (dd, *J* = 6.97, 11.15 Hz, 1H), 4.39 (q, *J* = 7.03 Hz, 2H), 1.36 (t, *J* = 7.10 Hz, 3H). **UIAA-II-193b** (**16b**) ^{1}H NMR (300 MHz, DMSO) δ = 12.45 (bs, 1H), 9.37 (dd, J = 8.41, 12.43, 1H), 8.47 (s, 1H), 8.39 (dd, *J* = 8.53, 10.78 Hz, 1H), 4.39 (q, *J* = 7.03 Hz, 2H), 1.36 (t, *J* = 7.10 Hz, 3H). **UIAA-II-193a-b** (**16a-b**) ^{19}F NMR (282 MHz, DMSO) δ = −132. 85 (ddd, *J* = 8.77, 10.69, 20.04 Hz, 1F), −143.32 (ddd, *J* = 7.32, 10.77, 17.06 Hz, 1F). HRMS (ESI) calculated for (M + H^+) 294.0685, found 294.0691. Retention time (analytical HPLC) **UIAA-II-193a** (**16a**) = 17.1 min and **UIAA-II-193b** (**16b**) = 18.2 min.

4.6.3. Preparation of ethyl 6-ethyl-8,9-difluoro-5-oxo-5,6-dihydropyrazolo [1,5c]quinazoline-2-carboxylate (17a) (UIAA-II-221)

UIAA-II-193a (**16a**) (0.080 g, 0.273 mmol) was dissolved in 2 mL of anhydrous DMSO under argon atmosphere, diisopropyl ethylamine (0.300 mL, 1.733 mmol) was added dropwise at room temperature. Consequently, iodoethane (0.077 mL, 0.958 mmol) was added, in one portion, and the resulting reaction mixture was heated to 40 °C for 41 h. The reaction mixture was then cooled to room temperature, gradually, and the pure product was precipitated out of solution with cold distilled water to afford pure **UIAA-II-221** (**17a**), as a yellow amorphous solid. Quantitative yield. ^{1}H NMR (300 MHz, DMSO) δ = 8.48 (dd, *J* = 8.58, 10.60 Hz, 1H), 7.90 (dd, *J* = 6.86, 12.95 Hz, 1H), 7.74 (s, 1H), 4.39 (q, *J* = 7.16 Hz, 2H), 4.31 (q, *J* = 7.05 Hz, 2H), 1.36 (t, *J* = 7.07 Hz, 3H), 1.28 (t, *J* = 6.25 Hz, 3H). ^{19}F NMR (282 MHz, DMSO) δ = −132.30 (m, 1F), −143.49 (m, 1F). HRMS (ESI) calculated for (M + Na^+) 344.0823, found 344.0811. Retention time (analytical HPLC) = 20.3 min.

4.6.4. Preparation of ethyl 6-ethyl-8,9-difluoro-5-oxo-5,6-dihydropyrazolo [1,5c]quinazoline-1-carboxylate (17b) (UIAA-II-230)

UIAA-II-193b (**16b**) (0.039 g, 0.133 mmol) was dissolved in 2 mL of anhydrous DMSO under argon atmosphere, diisopropyl ethylamine (0.075 mL, 0.433 mmol) was added dropwise at room temperature. Consequently, iodoethane (0.025 mL, 0.311 mmol) was added, in one portion, and the resulting reaction mixture was heated to 40 °C for 16 h. The reaction mixture was then cooled to room temperature, gradually, and the pure product was precipitated out of solution with cold distilled water to afford pure **UIAA-II-230** (**17b**). 36% yield. ^{1}H NMR (300 MHz, DMSO) δ = 9.50 (dd, *J* = 8.91, 12.38 Hz, 1H), 8.46 (s, 1H), 8.44 (dd, *J* = 8.58, 10.63 Hz, 1H), 7.45 (q, *J* = 7.45 Hz, 2H), 6.74 (q, *J* = 6.74 Hz, 2H), 1.36 (t, *J* = 7.11 Hz, 3H), 1.27 (t, *J* = 7.00 Hz, 3H). ^{19}F NMR (282 MHz, DMSO) δ = −132.30 (m, 1F), −143.49 (m, 1F). HRMS (ESI) calculated for (M + Na^+) 344.0823, found 344.0809. Retention time (analytical HPLC) = 23.9 min.

4.6.5. Preparation of (S)-ethyl 8-(3-(((tert-butoxycarbonyl)amino)methyl) pyrrolidin-1-yl)-6-ethyl-9-fluoro-5-oxo-5,6-dihydropyrazolo[1,5-c] quinazoline-2-carboxylate (18a) (UIAA-II-222)

To a stirring solution of **UIAA-II-221** (**17a**) (0.0730 g, 0.227 mmol) and (*R*)-*tert*-butyl (pyrrolidin-3-ylmethyl)carbamate (0.0921 g, 0.460 mmol), dissolved in 2 mL of anhydrous DMSO, triethylamine (0.255 mL, 1.814 mmol) was added, dropwise, at room temperature and under argon atmosphere. The reaction mixture was then heated to 60 °C for 20 h. Pure **UIAA-II-222** (**18a**) was precipitated from the cool reaction mixture with cold distilled water. 53% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.42 (d, *J* = 13.14 Hz, 1H), 7.09 (s, 1H), 6.38 (d, *J* = 7.21 Hz, 1H), 5.05 (m, 2H), 4.77(m, 1H), 4.48 (q, *J* = 7.12 Hz, 2H), 4.38 (q, *J* = 7.37 Hz, 2H), 3.66 (dd, *J* = 2.98, 7.42 Hz, 1H), 3.36 (dd, *J* = 2.81, 6.51 Hz, 1H), 3.28 (dd, *J* = 6.58, 12.71 Hz, 2H), 2.87 (dd, *J* = 7.31, 10.57 Hz, 1H), 2.17 (m, 1H), 1.82 (dd, *J* = 7.89, 12.15 Hz, 1H), 1.48 (s, 6H), 1.45 (s, 9H). ^{19}F NMR (282 MHz, $CDCl_3$) δ = −131.91 (s, 1F). HRMS (ESI) calculated for (M + Na^+) 524.2285, found 524.2281. Retention time (analytical HPLC) = 23.3 min.

4.6.6. Preparation of (S)-ethyl 8-(3-(((tert-butoxycarbonyl)amino)methyl) pyrrolidin-1-yl)-6-ethyl-9-fluoro-5-oxo-5,6-dihydropyrazolo[1,5-c] quinazoline-1-carboxylate (18b) (UIAA-II-231)

To a stirring solution of **UIAA-II-230** (**17b**) (0.0152 g, 0.0473 mmol) and (*R*)-*tert*-butyl (pyrrolidin-3-ylmethyl)carbamate (0.022 g, 0.110 mmol), dissolved in 1.5 mL of anhydrous DMSO, diisopropyl ethylamine (0.030 mL, 0.173 mmol) was added, dropwise, at room temperature and under argon atmosphere. The reaction mixture was then heated to 60 °C for 18 h. Precipitated crude **UIAA-II-231** (**18b**) with cold distilled water and washed with 15 mL of saturated NaCl solution. The organic layer was filtered through Na_2SO_4 and concentrated. The residue was then purified via flash chromatography utilizing an isocratic elution of 1:2 ethyl acetate:hexanes and then an isocratic elution of 100% ethyl acetate to afford pure **UIAA-II-231** (**18b**). 49% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 9.29 (d, *J* = 15.71 Hz, 1H), 8.37 (s, 1H), 6.34 (d, *J* = 7.52 Hz, 1H), 5.13 (bs, 1H), 4.80 (m, 1H), 4.40 (m, 4H), 3.69 (m, 2H), 3.62 (m, 2H), 3.27 (m, 2H), 2.56 (m, 1H), 2.17 (dd, *J* = 5.42, 11.22 Hz, 1H), 1.82 (dd, *J* = 7.72, 12.39 Hz, 1H), 1.48 (s, 9H), 1.27 (s, 6H), 0.88 (dd, *J* = 6.51, 13.92 Hz, 2H). ^{19}F NMR (282 MHz, $CDCl_3$ δ = −131.20 (s, 1F). HRMS (ESI) calculated for (M + H^+) 502.2460, found 502.2473. Retention time (analytical HPLC) = 28.1 min.

4.6.7. Preparation of (S)-ethyl 8-(3-(aminomethyl)pyrrolidin-1-yl)-6-ethyl-9-fluoro-5-oxo-5,6-dihydropyrazolo[1,5-c]quinazoline-2-carboxylate (19a) (UIAA-II-223)

UIAA-II-222 (**18a**) (0.0280 g, 0.0558 mmol) was dissolved in 4 mL of a 1:1 4 N HCl (aq.): acetonitrile mixture and was allowed to stir in ambient conditions for 4 h. The acetonitrile was removed from the reaction mixture by rotary evaporation and the resulting aqueous solution was frozen and lyophilized to afford pure **UIAA-II-223** (**19a**), as the HCl salt. Quantitative yield. ^{1}H NMR (300 MHz, DMSO) δ = 8.03 (d, *J* = 13.99 Hz, 1H), 7.48 (s, 1H), 6.54 (d, *J* = 7.54 Hz, 1H), 4.36 (m, 4H), 3.70 (m, 2H), 3.61 (m, 2H), 3.22 (m, 1H), 2.96 (bs), 2.21 (m, 2H), 1.70 (m, 2H), 1.33 (m, 6H). ^{19}F NMR (282 MHz, DMSO δ = −132.62 (s, 1F). HRMS (ESI) calculated for (M + Na^+) 424.1761, found 424.1749. Retention time (analytical HPLC) = 15.8 min.

4.6.8. Preparation of (S)-ethyl 8-(3-(aminomethyl)pyrrolidin-1-yl)-6-ethyl-9-fluoro-5-oxo-5,6-dihydropyrazolo[1,5-c]quinazoline-1-carboxylate (19b) (UIAA-II-232)

UIAA-II-231 (**18b**) (0.0011 g, 0.0219 mmol) was dissolved in 2 mL of a 1:1 4 N HCl (aq.): acetonitrile mixture and was allowed to stir in ambient conditions for 4 h. The acetonitrile was removed from the reaction mixture by rotary evaporation and the resulting aqueous solution was frozen and lyophilized to afford pure **UIAA-II-223** (**19b**), as the HCl salt. Quantitative yield. ^{1}H NMR (300 MHz, DMSO) δ = 9.19 (d, *J* = 16.20 Hz, 1H), 8.37 (s, 1H), 6.52 (m, 1H), 4.34 (m, 4H), 3.77 (m, 2H), 3.62 (m, 2H), 2.85 (bs), 2.16 (m, 2H), 1.79 (m, 2H), 1.29 (m, 6H), 0.85 (m, 1H). ^{19}F NMR (282 MHz, DMSO δ = −132.62 (s, 1F). HRMS (ESI) calculated for (M + H^+) 402.1936, found 402.1735. Retention time (analytical HPLC) = 16.4 min.

4.7. Scheme 5. Synthesis of the 5,6-dihydropyrazolo[1,5-c]quinazoline-2-carboxylic acid, **21**

4.7.1. Preparation of (S)-ethyl 8-(aminomethyl)pyrrolidin-1-yl)-6-ethyl-9-fluoro-5-oxo-5,6-dihydropyrazolo[1,5-c]quinazoline-2-carboxylic acid (21) (UIAA-II-226)

UIAA-II-222 (**16a**) (0.0350 g, 0.0698 mmol) was dissolved in 5 mL of NaOH (aq.) (0.0090 g, 0.2250 mmol) in ambient conditions. The reaction mixture was allowed to stir for 4 h, which was then consequently lyophilized to afford pure **UIAA-II-224** (**20**), which was used immediately in the next step. Retention time (analytical HPLC) = 19.3 min. **UIAA-II-224** (**20**) (0.0300 g, 0.0635 mmol) was dissolved in 6 mL of a 1:1 4 N HCl (aq.): acetonitrile mixture and was allowed to stir in ambient conditions for 2 h. The acetonitrile was removed from the reaction mixture by rotary evaporation and the resulting aqueous solution was frozen and lyophilized to afford pure **UIAA-II-226** (**21**), as the HCl salt. Quantitative yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 9.67 (bs, 1H), 7.99 (d, J = 13.76 Hz, 1H), 7.40 (s, 1H), 6.56 (J = 7.64 Hz, 1H), 4.33 (q, J = 6.83 Hz, 2H), 3.70 (m, 1H), 3.59 (m, 2H), 3.42 (m, 2H), 3.28 (t, 3H), 2.60 (m, 1H), 2.27 (m, 1H), 2.08 (m, 2H). HRMS (ESI) calculated for (M + H^+) 374.1623, found 374.1625. Retention time (analytical HPLC) = 12.0 min.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by National Institutes of Health (NIH) Research Grant R01 AI87671 to RJK. Arturo Aguirre and Pratik Chheda acknowledge training program support from the University of Iowa Center for Biocatalysis and Bioprocessing and the NIH-sponsored Predoctoral Training Program in Biotechnology (T32 GM008365).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2020.115439.

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