Design and synthesis of inhibitors of the ADP ribosylating toxin ExoS: Targeting the Type III Secretion System (T3SS) of *Pseudomonas aeruginosa*.

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Abstract

Pseudomonas aeruginosa, a gram-negative bacterium, is one of the most challenging pathogens due to intrinsic resistance to antibiotics. It has the ability to rapidly create new resistance mechanisms. Therefore, the lack of useful anti-pseudomonal agents forces us to develop new appropriate therapeutic agents via alternative strategies such as targeting the major virulence factors of the bacteria. These bacteria have a variety of virulence factors and one of them is type III secretion system (T3SS) which is the most attractive target for the anti-pseudomonal drug discovery. T3SS in *P. aeruginosa* is essential virulence factor that is responsible for the secretion of four effector proteins into the host cell which are ExotoxinS (ExoS), ExoT, ExoU, and ExoY. ExoS is a bifunctional enzyme and has a GTPase activating (GAP) domain and an ADP-ribosyl transferase (ADPRT) domain that are important for the pathogenicity of *P. aeruginosa* according to several studies. Herein, we target to inhibit ExoS ADPRT activity via small organic compounds as a new therapeutic strategy. For this reason, we designed and synthesized a set of compounds to investigate the role of the different side chains and scaffold to the activity against ADPRT activity of ExoS and also to improve activity. The side chain and main scaffold were modified with various groups that have different length and rigidity in order to establish structure-activity relationships (SAR) of compounds. Besides, all designed and synthesized compounds were tested against ExoS-ADPRT activity in an enzymatic assay which was developed by our collaborators in Karolinska Institute.

Keywords

ADPRT, antibiotic, antibiotic resistance, anti-pseudomonal, bacterial virulence, enzymatic assay, ExotoxinS (ExoS), *Pseudomonas aeruginosa*, type III secretion system (T3SS).

List of Abbreviations

ADP	Adenosine diphosphate
ADPRT	ADP-ribosyltransferase
Cdc42	Cell division control protein 42 homolog
CF	Cystic Fibrosis
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
eq	Equivalent
εNAD^+	1,N^6 -etheno NAD
EtOAC	Ethyl acetate
EtOH	Ethanol
ExoS	*P. aeruginosa* exotoxin S
ExoT	*P. aeruginosa* exotoxin T
ExoU	*P. aeruginosa* exotoxin U
ExoY	*P. aeruginosa* exotoxin Y
GAP	GTPase activating protein
IC_{50}	Half maximal inhibitory concentration
HPLC	High-performance liquid chromatography
M	Molar concentration
MLD	Membrane localization domain
MS	Mass Spectrometry
NAD	Nicotinamide adenine dinucleotide
P. aeruginosa	*Pseudomonas aeruginosa*
QS	Quorum Sensing
Rf	Retention factor
TBAI	1-(*p*-Toluenesulfonyl)imidazole
TEA	Triethylamine
TLC	Thin layer chromatography
TsIm	Tetrabutylammonium iodide
T3SS	Type III Secretion System
UV	Ultraviolet

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1. Introduction

1.1. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a gram-negative bacterium and an opportunistic human pathogen that causes serious infections and can be fatal among immunocompromised individuals such as HIV, cancer and organ transplant patients and infants who have an undeveloped immune system.[1] It is species of *Pseudomonas* and grows on a wide range of environments.[2] The first genome sequence of the wild type of these bacteria (PA01) was completed in 2000 and provided useful information which showed the bacteria contains various paralogous gene families encoding significant adaptive functions.[3] Genetic capacity of *P. aeruginosa* makes the bacteria capable of adapting to extreme environmental conditions.[3] Generally, it exists in water, air, soil, skins of animals, plants and humans.[4] Mostly, it lives in moist habitat. These bacteria are motile via polar flagella. *P. aeruginosa* strains play an significant role in nature during the decomposition and biodegradation of the organic materials and also carbon and nitrogen cycle. They are known as an aerobe but also can survive under anaerobic condition. It has been shown that they are highly adaptive within various enviromental alterations.[5]
P. aeruginosa has natural ability to develop resistance against all marked antibiotic classes.[6] For that treatment of *P. aeruginosa* infection remains challenging.[5] Clinically, infections of *P. aeruginosa* is very important due to high rates of drug resistance and mortality among patients with burn wounds, cystic fibrosis (CF) and compromised immunity.[2] It infects a numerous animal, plant and person with immunodeficiency.[4] In addition, *P. aeruginosa* infection may occur within healthy individuals, as well.[7] It is a healthcare-associated infection which can be acquired from contaminated sources and transmission of person to person.[1]
This pathogen has numerous virulence factors that are responsible for its pathogenesis.[8] Pili and flagella, quorum sensing (QS), Exotoxin A, the type III secretion system, phospholipase, and pyoverdin are the most important virulence factors of this pathogen. Except pili and flagella other virulence factors that mentioned above, contribute to cell and tissue destruction of host and also responsible for impaired host defences. Pili and flagella allow host cell adherence, motility, and biofilm formation.[9]

1.2 Drug-Resistance of *Pseudomonas aeruginosa*

The treatment of *P. aeruginosa* infections is extremely problematic because of the intrinsic resistant to a broad range of antibiotics.[6] It can easily develop resistance by means of several mechanisms which are associated with low membrane permeability, efflux pump and genetic mutations.[10] Multi-factorial mechanism of antibiotic resistance in *P. aeruginosa* is based on low outer membrane permeability for the antibiotics, multiple antibiotic modifying enzymes like ß-lactamases, metallo-ß-lactamases and aminoglycoside modifying enzyme, efflux pumps. Such as, MexAB-OprM, MexEF-OprN, MexCD-OprJ and MexXY-OprM and acquisition of encoded antibiotic resistance genes via plasmid and chromosomal mutations.[6] The first resistance mechanism is low outer membrane permeability, which serves to decrease the rate of uptake of most antibiotics and the second mechanisms is energy dependent multidrug efflux and ß-lactamase.[11]
The resistance rates of *P. aeruginosa* strains are significantly higher than the other gram-negative pathogens because it has large and versatility genome which are able to develop new resistance mechanisms to antibiotics and also contributes its pathogenicity. They also cause higher rate of mortality compare to other gram negative bacteria.[7]
The therapy for *P. aeruginosa* infections is more challenging since it has also multi-drug resistance which means the bacteria has resistance more than two of the antibiotics that were once effective for the combating of infectious. Multi-drug

resistance is making combination therapy of *P. aeruginosa* infections useless, difficult and unsuccessful.[12] Unfortunately, extensive use of antibiotics are leading to increase the amount of drug-resistant strains of *P. aeruginosa*.[11]
Hopefully, there are several alternative approaches in order to overcome difficulties in treating *P. aeruginosa* infections.

1.3 The Type III Secretion System and Exotoxin S

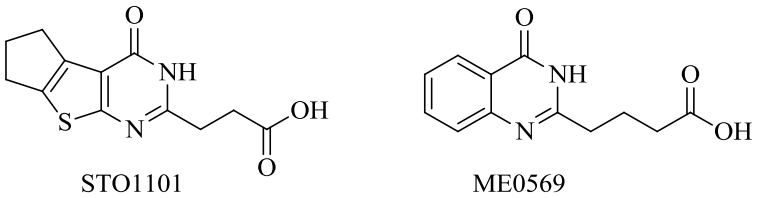
There are numerous virulence factors that contribute to the pathogenicity of *P. aeruginosa*. A major virulence factor of *P. aeruginosa* is the type III secretion system (T3SS) that is responsible of secretion effector toxins.[13] These toxins are ExotoxinS (ExoS), ExoT, ExoU and ExoY which play an important roles in the pathogenesis of the bacteria.[13b] T3SS is a needle-like complex structure and has five groups of proteins, which are the needle complex, the translocation apparatus, regulator proteins, chaperons and effector toxins.[13b, 14]
Only four effector proteins have been identified in T3SS of *P. aeruginosa*. Most strains of *P. aeruginosa* do not have the four effector encoding genes.[14] For this reason, strains of *P. aeruginosa* have either ExoS or the ExoU gene but not both of them. This characteristic feature can help to define phenotype of strains during the infection.[14]
ExoS is a bifunctional toxin and has GTPase activating protein (GAP) activity and adenosine diphosphate ribosyl transferase (ADPRT) activity.[15] The amino-terminus ExoS possess membrane localization domain (MLD) that is responsible for the temporary localization of ExoS to the plasma membrane of the host cell.[14] This localization of ExoS is very important for the useful modification of its substrates. Targets of GTP domain of ExoS are Rho, Rac and Cdc42.[14] GTP domain of ExoS causes disruption of the actin cytoskeleton of the host cell. ADPRT domain of ExoS has a wide number of negative effects on the host cell such as cell death, disruption of the actin cytoskeleton via cell rounding, inhibition of DNA synthesis and endocytosis. Both domain of ExoS lead to irreversible damage to the host cell by disruption of the cytoskeleton.[13b, 14-16]

1.4 STO1101 and ME0569: Inhibitors of ExoS-ADPRT

A recent study published by our group in collaboration with researchers at Karolinska Institute has identified **STO1101** and **ME0569** (Figure 1) as potent inhibitors of ExoS-ADPRT.[17]

STO1101 is a competitive inhibitor of ExoS with an IC_{50} value of 19 µM. In the structure of this molecule, there are two adjacent rings which are pyrimidone and cyclopente[b]thiophene and this ring system was substituted with propionic acid.

The IC_{50} value of **ME0569** is 25 µM and it has quinazolinone ring which substituted with butyric acid.[17]



STO1101 IC_{50}= 19 µM

ME0569 IC_{50}= 25 µM

Figure 1. Structure of **STO1101** and **ME0569**.

1.5 Aim of the diploma work

The main aim of the study was to establish structure-activity relationship (SAR) of the identified inhibitors of ExoS ADPRT (**STO1101**, **ME0569**).[17]

We also aimed to improve the potency of the identified inhibitors of ADPRT activity of ExoS as well as the pharmacokinetics and pharmacodynamics properties.

2. Popular scientific summary including social and ethical aspects

2.1 Popular scientific summary

Pseudomonas aeruginosa is one of the most common pathogen that causes of serious infections such as pneumonia, meningitis, soft tissue infections, chronic lung infections and corneal infections. *P. aeruginosa* infections associated with high rate of morbidity and mortality especially among immunocompromised individuals such as cystic fibrosis (CF), burn wound, or cancer.[11] Due to intrinsic resistance to a broad range of antibiotics, *P. aeruginosa* infections are very difficult to eradicate compared with other gram-negative pathogens infections.[6] Currently, *P. aeruginosa* infections may be treated by a combination of anti-pseudomonal agents in order to tackle resistance issue. However, this standard combination therapy remains problematic and not effective, as it leads to the increase of the drug-resistant strains.[18] Therefore, there are not many useful anti-pseudomonal drugs, *P. aeruginosa* infections are still one of the most dangerous infection disease in clinic. To combat the difficulties in treating *P. aeruginosa* infections, there are several approaches to develop new anti-pseudomona drugs. Targeting virulence factors, the ability of the bacteria to causes disease, is one of those novel strategies to fight against antibiotic resistance by disarming the bacteria.[11] Virulence factors of *P. aeruginosa* are responsible for the severity of infections because they cause irreversible host cell damage.[11] Especially exotoxins and proteases are associated with cell and tissue damage by disrupting the cytoskeletal structure and to develop of chronic infections.[11] *P. aeruginosa* has five protein secretion systems and among them, the type III secretion system transfers toxins (ExoS-T-U-Y) into the host cell.[15] ExoS is a bifunctional enzyme and possesses a GTPase-activating protein (GAP) activity and a ADP-ribosyl transferase (ADPRT) activity.[15] These activities work to disrupt the actin cytoskeleton and cause to cell death. The ADPRT domain of ExoS is responsible for the irreversible cell damage and it is highly toxic to cultured cells.[15]

2.2 Social and ethical aspects

Infection diseases are still major problem of clinic and *P. aeruginosa* causes severe and life-threating infections. Infections of *P. aeruginosa* are clinically challenged since it can readily develop antibiotic resistance during the therapy.[18] If the resistance occurs during the therapy, it will cause the length of hospital stay, additional medical procedures, surgery, chronic care and overall cost of antibiotics and even it will lead to the death of patients.[18] The high rate of drug-resistant strains of *P. aeruginosa* is increasing health threats facing the nation.

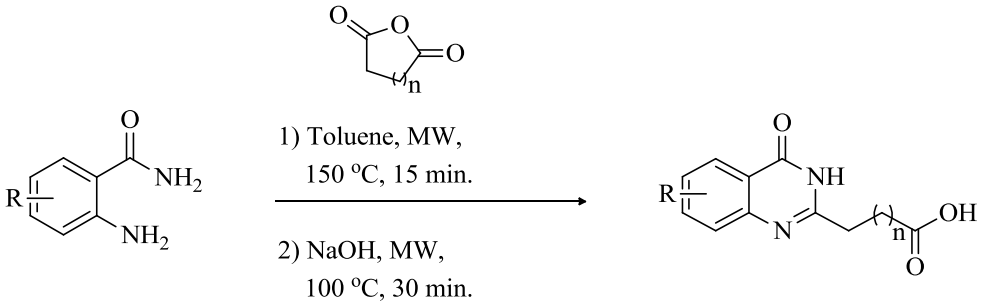
For this reason, there is an urgent need to develop new therapeutic agents against this pathogen for the combating *P. aeruginosa* infections. This project will help us to develop a new effective chemical probe which will be used as a chemical tool for the discovery of anti-pseudomonal drugs. Because of that this project is very important for the public health.

3. Experimental

3.1 Chemistry Section

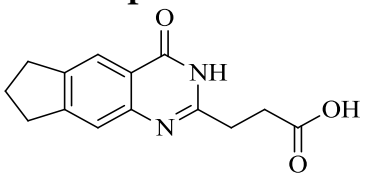
General. All reactions were carried out under nitrogen atmosphere. Chemicals and reagents were purchased from Aldrich, Alfa Aesar, AK Scientific, Matrix Scientific, or Apollo Scientific. Organic solvents were dried using the dry solvent system (Glass Contour Solvent Systems, SG Water USA) except CH_3CN, EtOH and $PhCH_3$, which were dried over activated molecular sieves 3 Å or 4 Å. Flash chromatography was performed on Biotage Isolera One using appropriate SNAP Cartridge KP-Sil or SNAP Ultra HP-Sphere 25µm Cartridge and UV absorbance at 254 nm. TLC was performed on Silica gel 60 F_{254} (Merck) with detection by UV light unless staining solution is mentioned. Preparative HPLC separation were performed on Gilson System HPLC, using a VP 250/21 NUCLEODUR C_{18} column HTEC 5 µm with a flow rate 18 mL/min, detection at 214 nm and eluent system: A. aq. 0.075% HCOOH, and B. 0.075% HCOOH in CH_3CN. The NMR spectra were recorded at 298 K on Bruker-DRX 400 MHz and 600 MHz using the residual peak of the solvent DMSO-d_6 (δ_H 2.50 ppm) or $CDCl_3$ (δ_H 7.26 ppm) as internal standard for ^1H, and DMSO-d_6 (δc 39.50 ppm) and CDCl3 (δ_c 77.16 ppm) as internal standard for ^{13}C. LCMS were recorded by detecting positive/negative ion (EC^+/EC^-) with an electrospray Water Micromass ZG 2000 instrument using XTerra MS C_{18} (5 µm 19x50 mm column) and H_2O/CH_3CN (0.2% HCOOH) as the eluent system, or with Agilent 1290 infinity – 6150 Quadrupole using YMC Triart C_{18} (1.9 µm 20x50 mm column) and H_2O/CH_3CN (0.1% HCOOH) as the eluent system.

General Synthetic procedure[19]



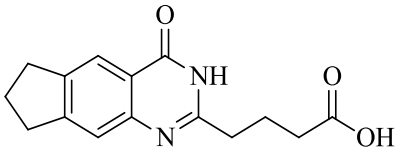
Compounds **ME0820** to **ME0828** were synthesized according to the above general procedure reported earlier by Öznur Aglar's degree report 2015 (**Appendix II** Method B)[19]

3-(4-oxo-4,6,7,8-tetrahydro-3H-cyclopenta[g]quinazolin-2-yl)propanoic acid- Compound 1 - ME0820 (300a_36)



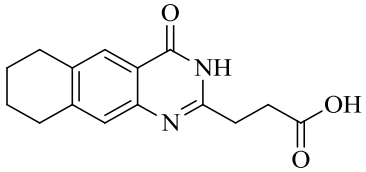
Synthesis: Methods B (3 % yield) **Chromatography:** A: aq. 0.75% HCOOH in H_2O, B: aq. 0.75% HCOOH in CH_3CN, organic phase gradient 25→100% over 25 min, white foam. **^{1}H NMR** (600MHz, DMSO-d_6): δ_H 12.67 (s, 1H), 12.14 (s, 1H), 7.88 (s, 1H), 7.40 (s, 1H), 2.96 (p, *J*= 7.8Hz, 4H), 2.82 (t, *J*= 6.6Hz, 2H), 2.71 (t, *J*= 6.6Hz, 2H), 2.06(p, 7.4, 2H) ppm. **^{13}C NMR** (150MHz, DMSO-d_6): δ_C 174.08, 162.26, 152.04, 148.26, 142.94, 127.29, 122.36, 120.86, 119.72, 33.02, 32.22, 30.50, 29.51, 25.79 ppm. **LC-MS** *m/z* calcd for $C_{14}H_{14}N_2O_3$ 258.27 [M+H^+]; 259.3 observed**.**

2-(4-oxo-3,4-dihydroquinazolin-2-yl)benzoic acid- Compound 2 - ME0822 (300a_38)



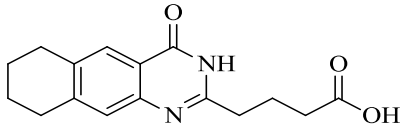
Synthesis: Methods B (4 % yield) **Chromatography:** A: aq. 0.75% HCOOH in H_2O, B: aq. 0.75% HCOOH in CH_3CN, organic phase gradient 25→100% over 25 min white foam. **^{1}H NMR** (600MHz, DMSO-d_6): δ_H 12.10 (s, 2H), 7.88 (s, 1H), 7.43 (s, 1H), 2.96 (p, *J*= 7.1Hz, 4H), 2.61 (t, *J*= 7.4Hz, 2H), 2.28 (t, *J*= 7.3Hz, 2H), 2.06 (p, *J*= 7.3Hz, 2H), 1.94 (p, *J*= 7.4Hz, 2H) ppm. **^{13}C NMR** (150MHz, DMSO-d_6): δ_C 174.65, 162.39, 156.20, 152.01, 142.93, 122.33, 120.78, 119.66, 33.919, 33.52, 33.03, 32.23, 25.81, 22.43 ppm. **LC-MS** *m/z* calcd for $C_{15}H_{16}N_2O_3$ 272.30 [M+H$^+$]; 273.3 observed.

3-(4-oxo-3,4,6,7,8,9-hexahydrobenzo[g]quinazolin-2-yl)propanoic acid- Compound 3- ME0823 (300a_39)



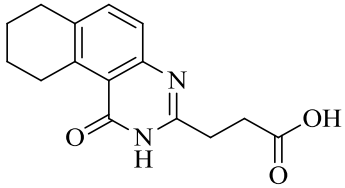
Synthesis: Methods B (2 % yield) **Chromatography:** A: aq. 0.75% HCOOH in H_2O, B: aq. 0.75% HCOOH in CH_3CN, organic phase gradient 25→100% over 25 min, white foam. **^{1}H NMR** (600MHz, DMSO-d_6): δ_H 12.20 (s, 2H), 7.75 (s, 1H), 7.27 (s, 1H), 2.87-2.83 (m, 4H), 2.80 (t, *J*= 6.9Hz, 2H), 2.69 (t, *J*= 6.9Hz, 2H), 1.76 (appear as p, *J*= 3.1Hz, 4H) ppm. **^{13}C NMR** (150MHz, DMSO-d_6): δ_C 174.15, 162.01, 155.92, 146.93, 144.68, 135.83, 126.61, 125.68, 119.106, 30.65, 29.65, 29.61, 29.00, 22.98, 22.72 ppm. **LC-MS** *m/z* calcd for $C_{15}H_{16}N_2O_3$ 272.30 [M+H$^+$]; 273.3 observed.

4-(4-oxo-3,4,6,7,8,9-hexahydrobenzo[g]quinazolin-2-yl)butanoic acid- Compound 4 - ME0824 (300a_40)



Synthesis: Methods B (2 % yield) **Chromatography:** A: aq. 0.75% HCOOH in H_2O, B: aq. 0.75% HCOOH in CH_3CN, organic phase gradient 25→100% over 25 min., white foam. **^{1}H NMR** (600MHz, DMSO-d_6): δ_H 12.07(s, 2H), 7.76 (s, 1H), 7.30 (s, 1H), 2.87-2.84 (m, 4H), 2.60 (t, *J*= 7.4Hz, 2H), 2.28 (t, *J*= 7.3Hz, 2H), 1.93 (p, *J*= 7.3Hz, 2H), 1.76 (appear as p, *J*= 3.1Hz, 4H) ppm. **^{13}C NMR** (150MHz, DMSO-d_6): δ_C 174.64, 162.18, 156.34, 147.12, 144.67, 135.84, 126.64, 125.65, 119.08, 33.93, 33.55, 29.64, 29.01, 22.98, 22.73, 22.37 ppm. **LC-MS** *m/z* calcd for $C_{16}H_{18}N_2O_3$ 286.33 [M+H$^+$]; 287.3 observed.

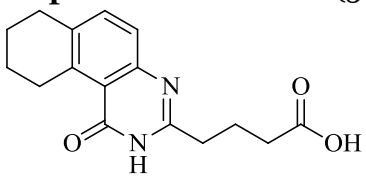
3-(1-oxo-1,2,7,8,9,10-hexahydrobenzo[f]quinazolin-3-yl)propanoic acid - Compound 5 - ME0825 (300a_41)



Synthesis: Methods B (5 % yield) **Chromatography:** A: aq. 0.75% HCOOH in H_2O, B: aq. 0.75% HCOOH in CH_3CN, organic phase gradient 25→100% over 25 min, white foam. **^{1}H NMR** (600MHz, DMSO-d_6): δ_H 12.46 (s, H), 11.95 (s, 2H), 7.39 (d, *J*= 8.3Hz, 1H), 7.27 (d, *J*= 8.2Hz, 1H), 3.35- 3.33 (m, 2H), 2.79- 2.76 (m, 4H), 2.69 (t, *J*= 7.0Hz, 2H), 1.75- 1.68 (m, 4H) ppm. **^{13}C NMR** (150MHz, DMSO-d_6): δ_C 174.06,

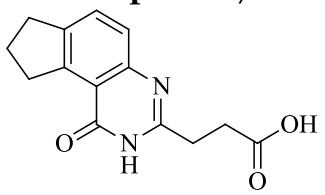
163.08, 155.61, 149.20, 138.74, 135.63, 135.008, 124.68, 119.16, 30.39, 30.17, 29.09, 28.29, 23.18, 22.15 ppm. **LC-MS** *m/z* calcd for $C_{15}H_{16}N_2O_3$ 272.30 [M+H$^+$]; 273.30 observed.

4-(1-oxo-1,2,7,8,9,10-hexahydrobenzo[f]quinazolin-3-yl)butanoic acid- Compound 6 - ME0826 (300a_42)



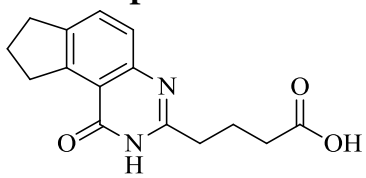
Synthesis: Methods B (14 % yield) **Chromatography:** A: aq. 0.75% HCOOH in H_2O, B: aq. 0.75% HCOOH in CH_3CN, organic phase gradient 25→100% over 25 min, white foam. **^{1}H NMR** (600MHz, DMSO-d_6): δ_H 11.91 (s, 1H), 7.39 (d, *J*= 8.3Hz, 1H), 7.29 (d, *J*= 8.2Hz, 1H), 3.35-3.32 (m, 2H), 2.78 (t, *J*= 5.8Hz, 2H), 2.55 (t, *J*= 7.4Hz, 2H), 2.26 (t, *J*= 7.3Hz, 2H), 1.91 (p, J= 7.4Hz, 2H), 1.76- 1.66 (m, 4H) ppm. **^{13}C NMR** (150MHz, DMSO-d_6): δ_C 174.66, 163.22, 156.23, 149.41, 138.71, 135.62, 134.98, 124.71, 119.15, 33.53, 33.50, 30.18, 28.94, 23.19, 22.37, 22.16 ppm. **LC-MS** *m/z* calcd for $C_{16}H_{18}N_2O_3$ 286.33 [M+H$^+$]; 287.3 observed.

3-(1-oxo-2,7,8,9-tetrahydro-1H-cyclopenta[f]quinazolin-3-yl)propanoic acid- Compound 7 - ME0827 (300a_43)



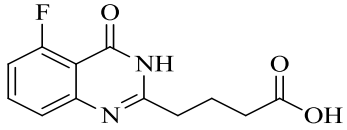
Synthesis: Methods B (14 % yield) **Chromatography:** A: aq. 0.75% HCOOH in H_2O, B: aq. 0.75% HCOOH in CH_3CN, organic phase gradient 25→100% over 25 min, white foam. **^{1}H NMR** (600MHz, DMSO-d_6): δ_H 12.41 (s, 1H), 7.55 (d, *J*= 8.1Hz, 1H), 7.30 (d, *J*= 8.1Hz, 1H), 3.35- 3.33 (m, 2H), 2.87 (t, *J*= 7.5Hz, 2H), 2.75 (t, *J*= 6.8Hz, 2H), 2.61 (t, *J*= 6.9Hz, 2H), 2.05 (p, *J*= 7.5, 2H) ppm. **^{13}C NMR** (150MHz, DMSO-d_6): δ_C 174.55, 162.58, 156.28, 148.90, 144.26, 142.32, 130.37, 125.46, 118.04, 34.05, 32.17, 31.51, 30.00, 25.40 ppm. **LC-MS** *m/z* calcd for $C_{14}H_{14}N_2O_3$ 258.27 [M+H$^+$]; 259.3 observed.

4-(1-oxo-2,7,8,9-tetrahydro-1H-cyclopenta[f]quinazolin-3-yl)butanoic acid- Compound 8 - ME0828 (300a_44)



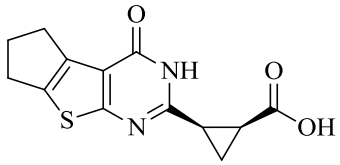
Synthesis: Methods B (11 % yield) **Chromatography** A: aq. 0.75% HCOOH in H_2O, B: aq. 0.75% HCOOH in CH_3CN, organic phase gradient 25→100% over 25 min, white foam. **^{1}H NMR** (600MHz, DMSO-d_6): δ_H 12.10 (s, 1H), 7.58 (d, *J* = 8.1Hz, 1H), 7.35 (appeared as d, *J* = 8.1Hz, 1H), 3.37- 3.35 (m, 2H), 2.89 (t, *J*= 7.5Hz, 2H), 2.57 (t, *J* = 7.4Hz, 2H), 2.25 (appear as t, *J* = 7.3Hz, 2H), 2.07 (p, *J* = 7.5Hz, 2H), 1.92 (p, *J* = 7.4Hz, 2H) ppm. **^{13}C NMR** (150MHz, DMSO-d_6): δ_C 174.80, 162.70, 156.39, 148.98, 144.25, 142.39, 130.42, 125.55, 117.99, 34.04, 33.88, 33.82, 32.18, 25.42, 22.59 ppm. **LC-MS** *m/z* calcd for $C_{15}H_{16}N_2O_3$ 272.30 [M+H$^+$]; 273.3 observed.

4-(5-fluoro-4-oxo-3,4-dihydroquinazolin-2-yl)butanoic acid - 9 - ME0850 (300a_52)



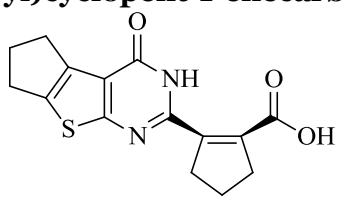
Synthesis: 2-amino-6-fluorobenzamide (150 mg, 0.97 mmol) and glutaric anhydride (5 equiv.) were suspended in toluene (0.25M, 3.88 ml) and irradiated to microwave at 150 degree for 30 minutes. After the end of the first step of reaction, NaOH (2M, 20 equiv.) was added to the reaction mixture and irradiated to microwave at 100 ^{0}C for 30 minutes. The each reaction's steps were monitored by TLC and LCMS. The reaction mixture was washed with NaOH (1M). The aqua phase was collected and acidified with HCl (6M) until the formation of precipitates. The precipitates were collected by filtration and washed with water and evaporated. The product was dried under vacuo as a pure white powder (69 % yield). **^{1}H NMR** (600MHz, DMSO-d_6): δ_H 12.19- 12.10 (m, 2H), 7.75- 7.70 (m, 1H), 7.40 (appeared as d, J = 7.4Hz, 1H), 7.18 (t, J= 8.9Hz, 1H), 2.60 (t, J= 7.2Hz, 2H), 2.30 (t, J = 6.9Hz, 2H), 1.93 (appear as t, J = 7.1Hz, 2H) ppm. **^{13}C NMR** (150MHz, DMSO-d_6): δ_C 174.51, 160.97 (d, J_{CF}=262Hz), 159.54, 158.48, 151.52, 135.26 (d, J_{CF}=10Hz), 123.36, 112.76 (d, J_{CF}=20Hz), 110.84 (d, J_{CF}=6Hz), 33.78, 33.24, 22.12 ppm. **LC-MS** *m/z* calcd for $C_{12}H_{11}FN_2O_3$ 250.23 [M+H$^+$]; 251.2 observed**.** NMR data of the compound has been shown in **Appendix III**.

2-(4-oxo-4,5,6,7-tetrahydro-3H-cyclopenta[4,5]thieno[2,3-d]pyrimidin-2 yl) cyclopropanecarboxylic acid - Compound 10 - ME0876 (300a_63)



Synthesis: 2-Aminothiophene-3-carboxamide (100 mg, 0.54mmol) and 3-oxabicyclo[3.1.0]hexane-2,4-dione (307.55 mg, 2.74 mmol) were suspended in toluene (0.25M) and irradiated to microwave at 150 degree for 30 minutes. After the end of the first step of reaction, NaOH (2M, 20 equiv.) was added to the reaction mixture and irradiated to microwave at 100 ^{0}C for 30 minutes. The each reaction's steps were monitored by TLC and LCMS. The reaction mixture was washed with NaOH (1M). The aqua phase was collected and acidified with HCl (6M) until the formation of precipitates. The precipitates were collected by filtration and washed with water and evaporated. The product was dried under vacuo and the final product was purified by HPLC as a pure white powder. **^{1}H NMR** (600MHz, DMSO-d6): δH 12.52 (s, 1H), 12.17 (s, 1H), 2.91- 2.87 (t, J=7.2 Hz, 4H), 2.41- 2.34 (m, 3H), 2.06- 2.00 (m, 1H), 1.69- 1.65 (appeared as dd, J = 11.2Hz, 1H), 1.36 – 1.31 (m, 1H) ppm. **^{13}C NMR** (150MHz, DMSO-d6): **^{13}C NMR** 172.36, 168.57, 158.66, 139.80, 136.96, 118.79, 60.99, 30.78, 29.40, 29.12, 27.90, 23.22, 22.14 ppm. **LC-MS** m/z calcd for C13H12N2O3S 226.31 [M+H+]; 227.3 observed.

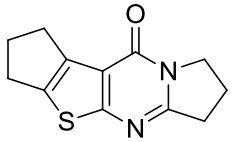
2-(4-oxo-4,5,6,7-tetrahydro-3H-cyclopenta[4,5]thieno[2,3-d]pyrimidin-2-yl)cyclopent-1-enecarboxylic acid - Compound 11 - ME0877 (300a_64)



Synthesis: 2-Aminothiophene-3-carboxamide (100 mg, 0.54mmol) and 1-cyclopentene-1,2-dicarboxylic anhydride (378.88 mg, 2.74 mmol) were suspended in toluene (0.25M) and irradiated to microwave at 150 degree for 30 minutes. After the end of the first step of reaction, NaOH (2M, 20 equiv.) was added to the reaction mixture and irradiated to microwave at 100 ^{0}C for 30 minutes. The each reaction's steps were monitored by TLC and LCMS. The reaction mixture was washed with NaOH (1M). The aqua phase was collected and acidified with HCl (6M) until the

formation of precipitates. The precipitates were collected by filtration and washed with water and evaporated. The product was dried under vacuum and the final product was purified by HPLC as a pure white powder. **^{1}H NMR** (600MHz, DMSO-d6): δH 13.29 (s, 1H), 2.91- 2.88 (t, J = 7.2Hz, 6H), 2.78- 2.74 (m, 2H), 2.40- 2.33 (appeared as p, J = 7.3Hz, 2H), 1.89 – 1.82 (p, J= 7.6Hz, 2H) ppm. **^{13}C NMR** (150MHz, DMSO-d6): δC 168.17, 167.63, 157.94, 150.02, 144.48, 140.28, 139.10, 119.63, 37.54, 36.31, 29.56, 29.07, 29.90, 21.27 ppm. **LC-MS** m/z calcd for C15H14N2O3S 302.35 [M+H+]; 303.3 observed.

2,3,7,8-tetrahydro-1H-cyclopenta[4,5]thieno[2,3-d]pyrrolo[1,2-a]pyrimidin-10(6H)-one – Compound 12- (300a_65)



Synthesis :A mixture, (3-hydroxypropyl)-6,7-dihydro-3H-cyclopenta[4,5]thieno[2,3-d]pyrimidin-4(5H)-one **ME07872** (0.01 mmol), TsIm (1.5 eq.), TEA (2 eq.), NaN_3 (3 eq.) and a catalytic amount of TBAI (1.1 mg) was refluxed in DMF (30 mL). Reflux was continued until TLC monitoring indicated no further improvement in the conversion. The solvent was evaporated under vacuum and the remaining foam was dissolved in DMSO (2 mL) and the crude product was purified by HPLC.
^{1}H NMR (600MHz, DMSO-d_6): δ_H 4.02 (t, J= 7.3Hz, 2H), 3.08 (t, J = 8.0Hz, 2H), 2.91 (t, J= 7.3Hz, 4H), 2.38 (p, J = 7.3Hz, 2H), 2.18 (p, J= 7.6Hz, 2H) ppm. **^{13}C NMR** (150MHz, DMSO-d_6): δ_C 168.80, 160.65, 157.01, 150.02, 139.42, 136.80, 117.75, 46.68, 32.04, 29.47, 29.16, 27.78, 19.63 ppm. **LC-MS** *m/z* calcd for $C_{12}H_{12}N_2OS$ 232.30 [M+H$^+$]; 233.1 observed**.**

3.2 Biology Section

3.2.1 Dose-response experiment:

All compounds were dissolved in DMSO to make a stock solution of 40mM, which was used to prepare a dilution series of 8 concentration points. The highest concentration was 200 μM and the lowest concentration was 1.562 μM. The dilution series was prepared in 96-well polypropylene compound plate, before it was transferred into the assay plate (Figure 2).

Protein master mix (ExoS + Ras + 14.3.3) which were obtained from research group of Herwig Schüler in Karolinska Institute was prepared including controls as following:

- Master mix without 14.3.3[17] : 0 % active enzyme
- DMSO: 100 % inactive enzyme
- STO1101: 30 μM concentration of inhibitor of ExoS ADPRT activity was used as a control.

Enzymatic assay:

- Ɛ-NAD+ is a fluorescent analog of NAD and used as a substrate for ExoS.
- Ras is a small GTPase protein subfamily and used as a co-substrate.
- 14.3.3 is a cofactor of ExoS and is required for activity of enzyme (ExoS).

The enzymatic assay is described in the discussion part.

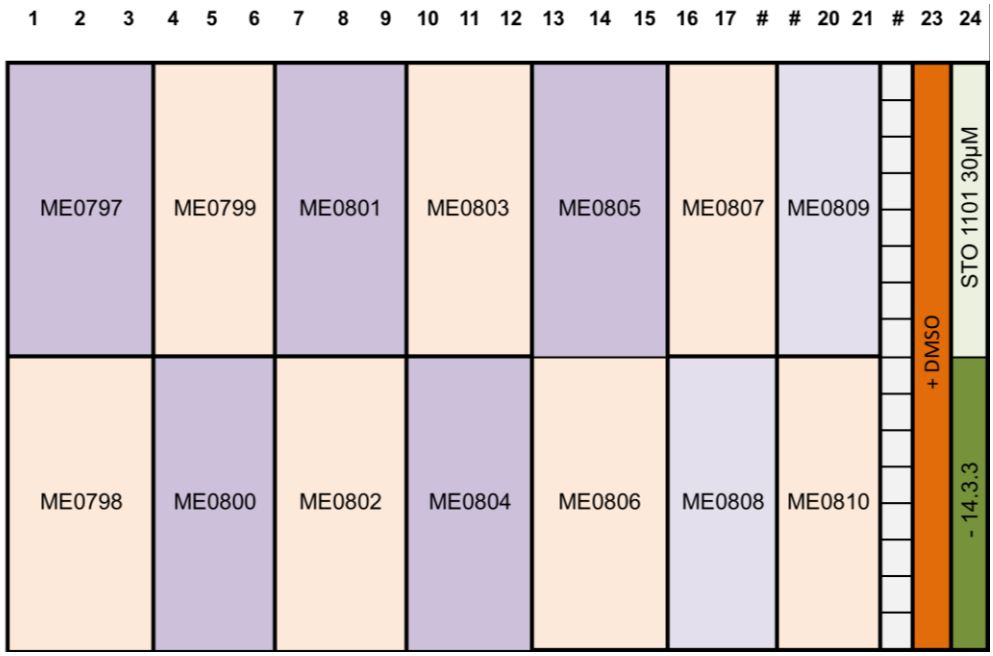


Figure 2. General dose-response assay format, in which compounds are screened against ExoS ADPRT activity in 384-well plates.

Table 1. Dose–response analysis of ExoS ADPRT activity inhibition. Dose–response curves of the compounds have been shown (**Appendix I)**.

Compound No	**IC_{50} (µM)**
ME0815	27
ME0816	585
ME0817	ND
ME0818	106
ME0819	32
ME0820	98
ME0821	141
ME0822	86
ME0823	ND
ME0824	ND
ME0825	ND
ME0826	ND
ME0827	54
ME0828	275
ME0850	25
ND: Not determined	

4. Results and Discussion

Due to antibiotic resistance problem, there is an urgent need for useful alternative therapeutics methods. There are several attractive approaches based on targeting bacterial virulence systems or disrupting interaction between the host cell and the bacteria.[20] T3SS which is responsible for the pathogenesis of the bacteria is one of the major virulence factors of *P. aeruginosa*. Our strategy is inhibiting ADPRT activity of ExoS of T3SS in *P. aeruginosa* via small organic compounds.

Recently, two potent inhibitors of ExoS-ADPRT have been described[17], which are **STO1101** and **ME0569** as shown in figure 1. Therefore, we designed a small library of analogues of both compounds. In this study, our attention was to synthesize analogues of **ME0569**.

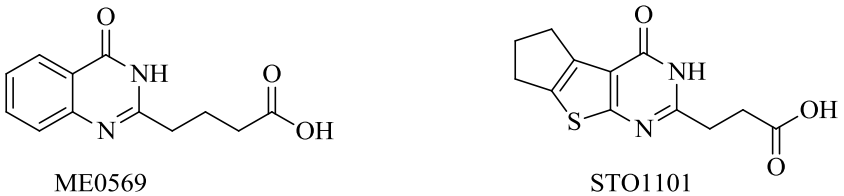
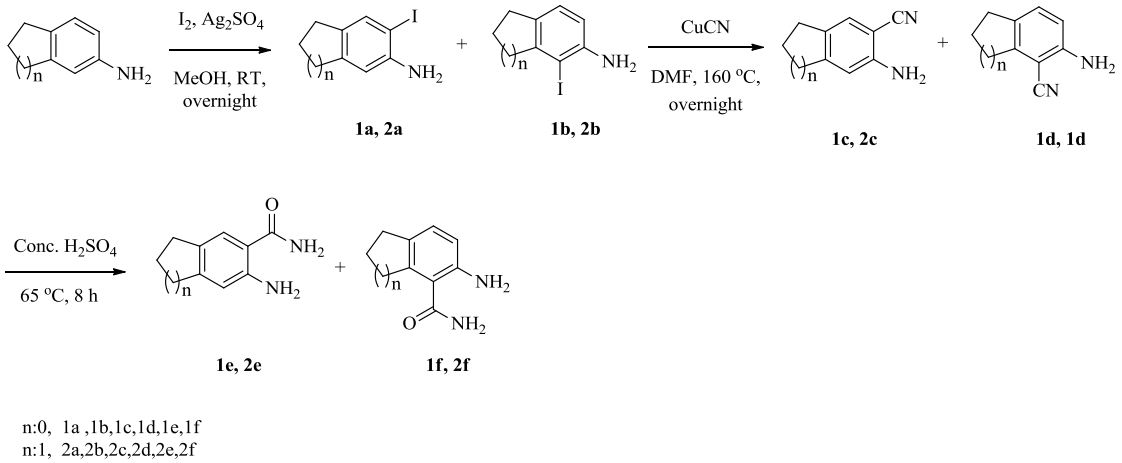


Figure 1. Structure of the **ME0569** and **STO1101**.

Primary structure-activity relationship (SAR) of the hit compounds (**STO1101** and **ME0569**) has been showed that carboxylic acid, amide proton, and pyrimidine ring are needed for the activity.[17] On this basis, we focused our efforts to investigate the side chain and the main scaffold of **ME0569**. For that, we designed 15 analogues in which the flexibility and the length of the side chain were investigated by modifying the length and the rigidity of the linker (Table 1). Different building blocks were selected including ether linker, hexyl and pentyl linkers, phenyl ring and furan ring. In comparing with **STO1101** we also decided to introduce different fused alkyl ring to the main scaffold of **ME0569**. For that the building blocks **1e**, **2e**, **1f** and **2f** were synthesized in 3 steps starting from iodination as shown in scheme 1 and the compounds **1a-f** and **2a-f** have been described in previous report.[19]



Scheme 1. The synthetic route to intermediates.[19]

Two reaction methods were performed to synthesize analogues (method A and B)[19] **(Appendix II)**. Method A is an efficient method in which corresponding aldehydes used one pot reaction in order to get the final product. Method B is two steps one pot microwave reaction in the presence of corresponding amides and anhydrides. Due to the solubility of the product and polarity of carboxylic acid, it was not possible to

purify the crude product by silica gel column chromatography. All compounds were purified by HPLC which resulted in low yield of the pure products.

For the synthesis of building blocks, the compounds **1a-f,** and **2a-f,** need to be synthesized because they were not commercially available. It was started from iodination and then nucleophilic substitution on the aromatic ring with nitrile and after that, hydrolysis of **1c-d** and **2c-d** for the synthesis of corresponding amides. In order to prepare the corresponding amides (**1e, 1f, 2e** and **2f**) several hydrolysis reactions were attempted with different reactions including Cs_2CO_3 (cesium carbonate), K_2CO_3 (potassium carbonate), K_2CO_3-peroxide and urea peroxide-K_2CO_3 and KOH (potassium hydroxide) and concentrated H_2SO_4. It turns out that, H_2SO_4 hydrolysis is an efficient method in very good yield.

Overall, a total of 15 analogues of **ME0569** were synthesized and tested in the enzymatic assay for their inhibition of ExoS-ADPRT activity. Dose-response experiments were performed to determine IC_{50} values of the compounds as described earlier in section 3.2.1 **(Appendix I)**.

The assay is based on measurement the increase in fluorescence intensity when the ADP-ribosyl moiety is transferred from εNAD$^+$ (a fluorescent analog of NAD) to the target protein Ras. For the enzyme activity a co-factor, 14.3.3 is required. εNAD$^+$ is a co-substrate, while, Ras is substrate. In the presence of 14.3.3, ExoS is an active enzyme and it catalyzes the cleavage of the glycoside bond of 1,N^6 -etheno NAD which results in a 10-fold increase in fluorescence intensity (Figure 3).

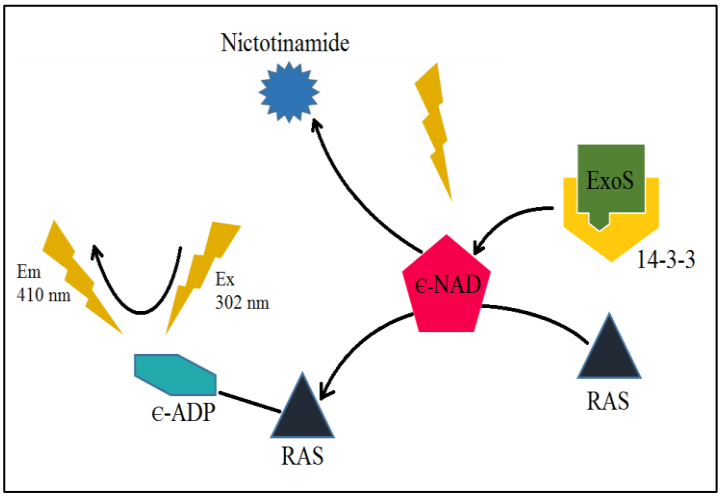


Figure 3. The scheme is illustrating the reaction in the enzymatic assay.

As a result, we earlier identified two compounds **ME0815** and **ME0850** that inhibited ExoS activity with IC_{50} values of 27 µM and 25 µM. However, the majority of the synthesized analogues did not allow us to improve the inhibition activity of this compound class. One clear issue with this class of compounds is their autofluorescence activity, which interferes with the assay measurement and a background correction had to be considered. Compound, **ME0850** a very close analogue to the lead compound **ME0569** has been proven to be as the lead compound. This result may imply that fluorine atom did not add any additional interaction with the target enzyme.

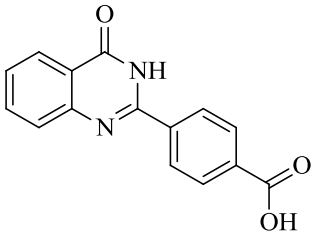
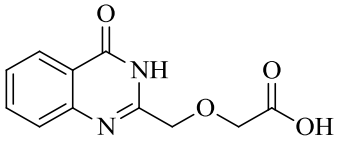
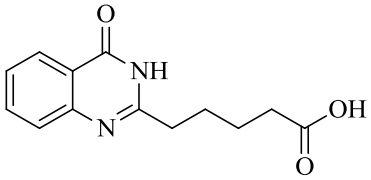
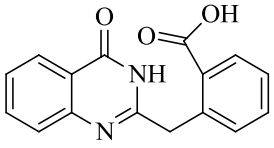
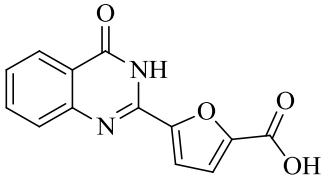
Nevertheless, structure-activity relationship (SAR) analysis (Table 2) allowed us to study the importance of the main features of the lead compound **ME0569**. We proved that longer chain (**ME0817**) was not favored as it reduces the inhibitory activity significantly (>100 µM). Also introducing a linker with hetero atom such as etheric oxygen (**ME0816**) did not contribute to any gained interaction with the target

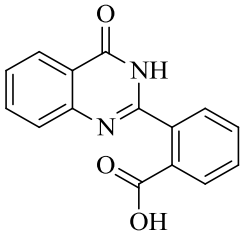
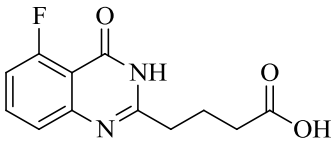
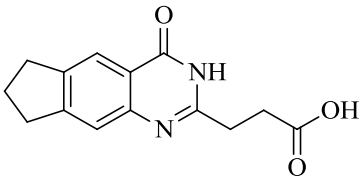
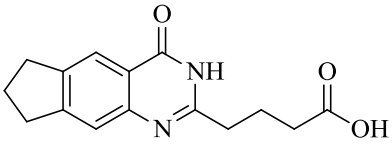
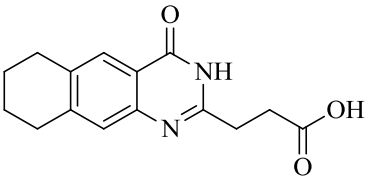
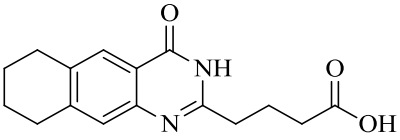
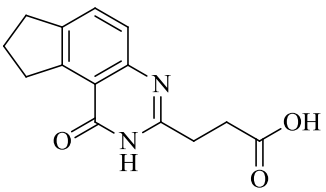
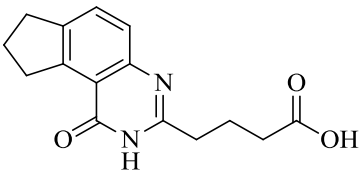
enzyme or any favored conformation change. A flexible linker was shown to be more favored. That might be due to conformational change and preferably intermolecular hydrogen bonding between the terminal carboxylic acid and the amide bond in the core scaffold. This result concludes that the need for a flexible linker with three carbon atoms between the carboxylic acid and the core scaffold. Quinazolinone ring, on the other hand, was substituted on different positions with fused alkyl rings including six- and five-membered rings. Our results have shown that a fused alkyl ring larger than 5-membered ring (i.e six-membered ring) was not tolerated, which confirm earlier results on the analogue **STO1101.**[17]

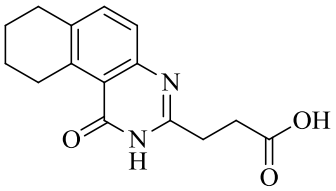
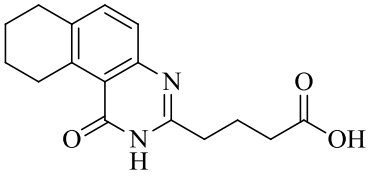
However, fused 5-membered ring compounds **ME0820**, **ME0822**, were tolerated, they did not show any improved activity. These results indicate a limited modification or substitutions pattern on the quinazolinone ring.

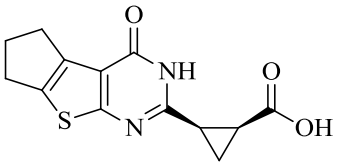
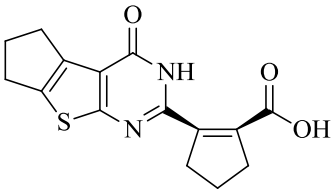
As parallel study on **STO1101** SAR has led to the identification of two more potent inhibitors **ME0800** (IC_{50}= 14.5 µM) and **ME0805** (IC_{50}= 10.6 µM) (data not shown). Therefore, our group decided to focus the effort trying to improve the activity of those two analogues of **STO1101**. Thereby, we designed and synthesized a small set of analogues in order to finalize this SAR with ExoS inhibitors (data are not shown). All promising inhibitors of ExoS-ADPRT will eventually be tested the cell-based assay.

Table 2. Structure-activity relationship analysis (SAR) of analogues.

Compound No	Structure of compound	IC_{50} (µM)
ME0815		27 (Appendix II)[19]
ME0816		>100 (Appendix II)[19]
ME0817		>100 (Appendix II)[19]
ME0818		>100 (Appendix II)[19]
ME0819		32 (Appendix II)[19]

ME0821		>100 (Appendix II)[19]
ME0850		25
ME0820		98
ME0822		86
ME0823		>100
ME0824		>100
ME0827		54
ME0828		>100

ME0825		>100
ME0826		>100

ME0876		Not tested
ME0877		Not tested

5. Conclusions

In the present study, we successfully designed and synthesized a total of 17 analogues of **STO1101** and **ME0569** for further development of inhibitors. All designed compounds were tested in the enzymatic assay and their IC_{50} values were determined via concentrated-response experiments. We identified two potent analogues (**ME0815** & **ME0850**) of **ME0569** as inhibitors of ExoS ADPRT activity with IC_{50} value of 27µM and 25µM respectively. Furthermore, all promising analogues will be tested in a cell-based assay for their activity.

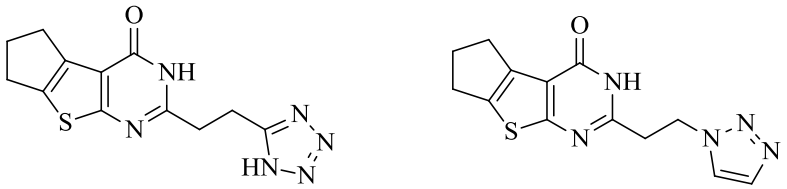
It was found that some analogues possess solubility problems in DMSO during the dose-response experiments and also most of the analogues fluoresce naturally. Dose-response experiments indicate that the solubility and theirs fluorescence properties in a fluorescence-based assay for ExoS activity are a significant challenge for such compounds.

In conclusion, this study allowed us to better understand the structural activity of this compound class, and to determine the important structural features such as the length of the linker and the substitution of the main scaffold.

Accordingly, we believe that these results make possible to develop a chemical probes, which can be used as a research tool to discover novel antibacterial agent against the *P. aeruginosa* infections.

6. Future Plan

A small set of potent hits (**ME0800** and **ME0805**) has been designed in order to improve potency of inhibitors and finalize SAR analyze of **STO1101**.[17] In this library, two compounds (**ME0876** and **ME0877**) were synthesized. In addition, two more compounds will be synthesized (Figure 4) and all synthesized compounds to be tested in enzymatic assay.

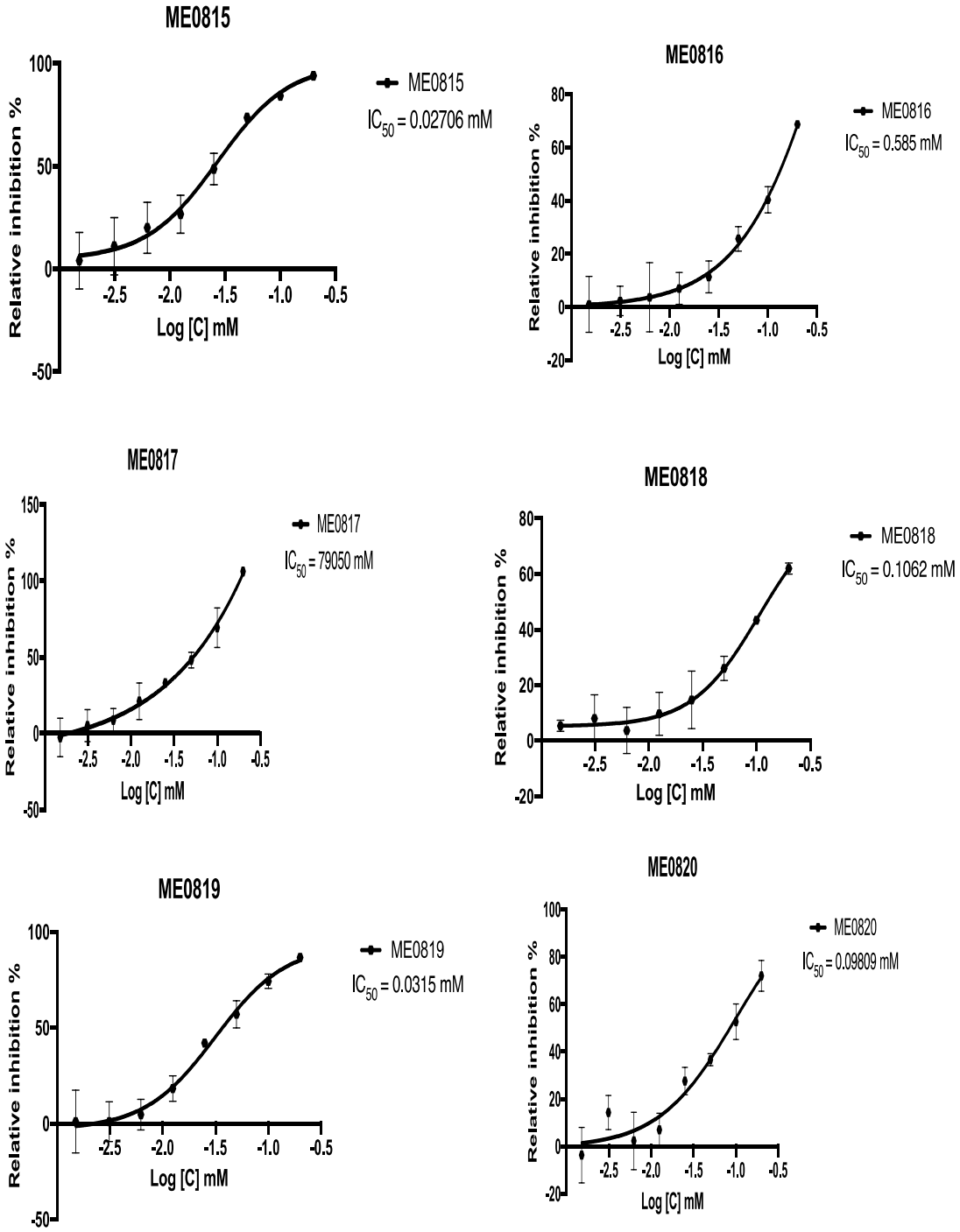


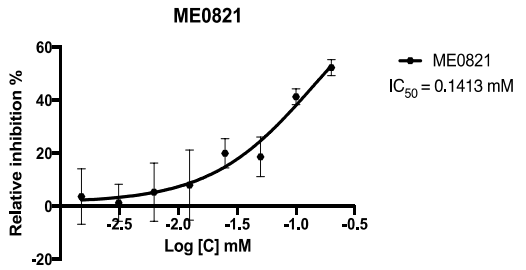
(a) **(b)**

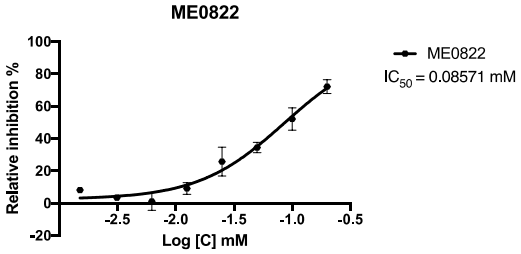
Figure 4. Structure of the compounds (a, b) that to be synthesized.

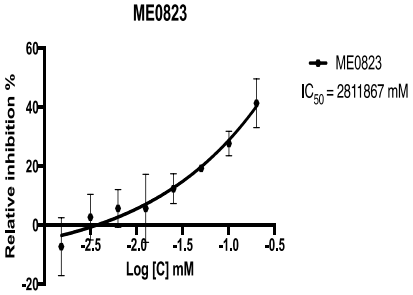
7. Appendix I

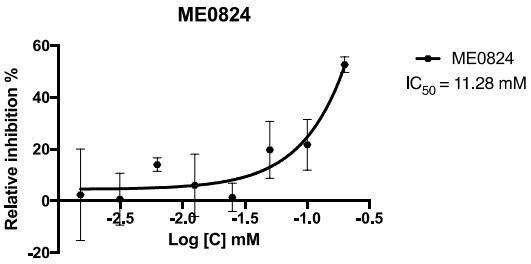
Concentration–response analysis of ExoS ADPRT activity inhibition. Compound numbers shown with corresponding dose–response curves. Data shown for ExoS ADPRT activity for promising compounds between the analogues: **ME0815** and **ME0850**.

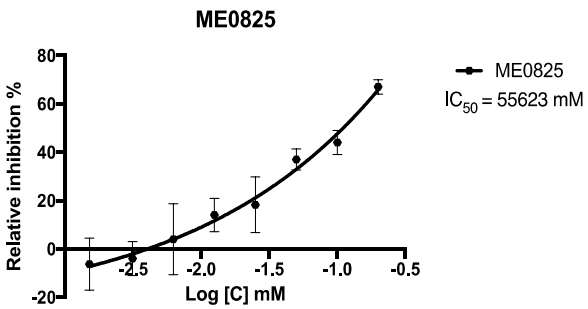


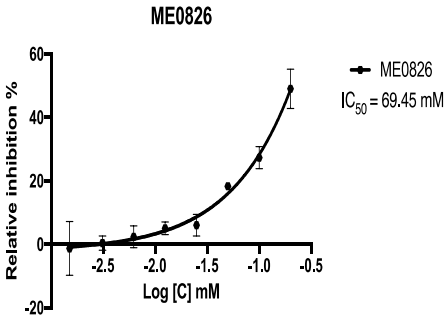

ME0821
Relative inhibition %
Log [C] mM
ME0821
IC50 = 0.1413 mM

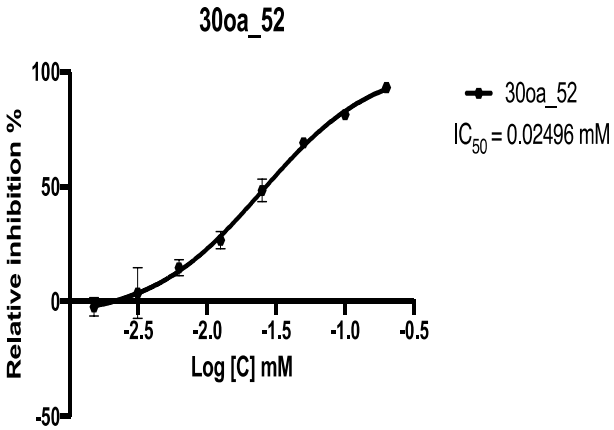

ME0822
Relative inhibition %
Log [C] mM
ME0822
IC50 = 0.08571 mM


ME0823
Relative inhibition %
Log [C] mM
ME0823
IC50 = 2811867 mM


ME0824
Relative inhibition %
Log [C] mM
ME0824
IC50 = 11.28 mM

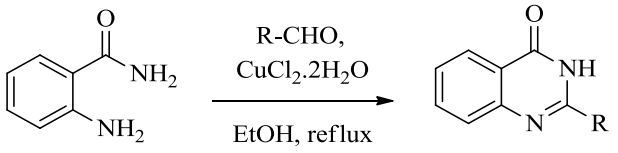

ME0825
Relative inhibition %
Log [C] mM
ME0825
IC50 = 55623 mM


ME0826
Relative inhibition %
Log [C] mM
ME0826
IC50 = 69.45 mM


30oa_52
Relative inhibition %
Log [C] mM
30oa_52
IC50 = 0.02496 mM

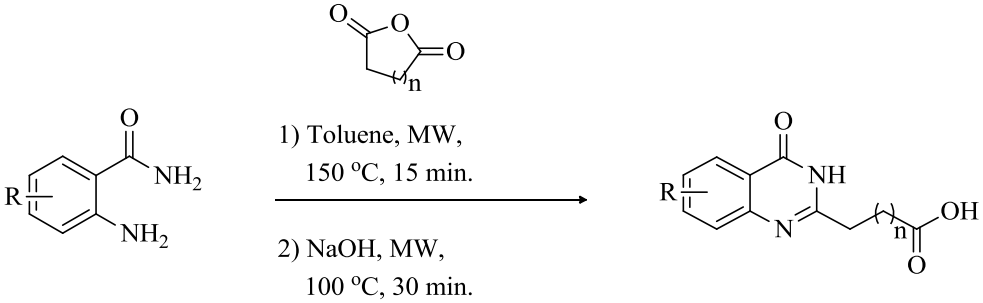
8. Appendix II

Method A:[21]



2-Aminobenzamide (0.50 mmol), corresponding aldehydes (0.525 mmol) and $CuCl_2.2H_2O$ (1 mmol) in dry ethanol (4ml) were refluxed for 16 hours. The reaction was monitored with TLC and LCMS. After completion of reaction, the reaction mixture was cooled to room temperature and distilled water was added until the formation of precipitates. The precipitates were filtered and washed with distilled water. Product was isolated as a white solid and dried under vacuo. Final product was purified by HPLC (A: aq. 0.75% HCOOH in H_2O, B: aq. 0.75% HCOOH in CH_3CN, organic phase gradient 25→100% over 25 min).

Method B:



Scheme 2 Synthesis of derivatives of quinazolinones via two steps one-pot reaction.

Derivatives of 2-aminobenzamide (0.9 mmol, 1 equiv.) was suspended in toluene (0.25M, 3.6 ml) in the presence of corresponding anhydride (5 equiv.) and irradiated to microwave at 150 °C for 30 minutes. The reaction mixture, was monitored by TLC and LCMS. Then it was concentrated and NaOH (2M, 20 equiv.) was added to the mixture and irradiated to microwave at 100 °C for 30 minutes. The reaction was monitored by TLC (Heptane/ EtOAc, 1:2) and LCMS till completion. After completion, the mixture was washed with NaOH (1M). The aqueous phase was collected and acidified with HCl (6M) till pH<7 and extracted with EtOAc, dried on anydrous Na_2SO_4 and evaporated. The product was dried under vacuo. HPLC was performed on the final products (A: aq. 0.75% HCOOH in H_2O, B: aq. 0.75% HCOOH in CH_3CN, organic phase gradient 25→100% over 25 min).

4-(4-oxo-3,4-dihydroquinazolin-2-yl)benzoic acid- ME0815 (300a_03)
Synthesis: Methods A (4 % yield) **Chromatography:** A: aq. 0.75% HCOOH in H_2O, B: aq. 0.75% HCOOH in CH_3CN, organic phase gradient 25→100% over 25 min., white foam. **^{1}H NMR** (600MHz, DMSO-d_6): δ_H 13.28 (s, 1H), 12.68 (s, 1H), 8.28 (appeared as d, J = 8.4Hz, 2H), 8.18 (appear as dd, J = 7.8Hz, 1.12Hz, 1H), 8.08 (d, J = 8.4Hz, 2H), 7.87 (ddd, J = 8.1Hz, 7.0Hz, 1.3Hz, 1H), 7.78 (appeared as d, J = 7.9Hz, 1H), 7.56 (ddd, J = 7.9Hz, 6.9Hz, 0.9Hz 1H) ppm. **^{13}C NMR** (150MHz, DMSO-d_6): δ_C 167.27, 162.59, 152.12, 149.03, 136.89, 135.19, 129.87, 128.50, 128.13, 127.45, 126.37, 121.60 ppm. **LC-MS** *m/z* calcd for $C_{15}H_{10}N_2O_3$ 266.25 [M+H$^+$]; 267.3 observed.

2-((4-oxo-3,4-dihydroquinazolin-2-yl)methoxy)acetic acid - ME0816 (300a_07)

Synthesis: Methods B **Chromatography:** A: aq. 0.75% HCOOH in H_2O, B: aq. 0.75% HCOOH in CH_3CN, organic phase gradient 25→100% over 25 min., white foam. **^{1}H NMR** (600MHz, DMSO-d_6): δ_H 12.226 (s, 1H), 12.91 (s, 1H), 8.119 (dd *J*=7.9Hz, 1.2Hz, 1H), 7.82 (ddd *J*=6.8Hz, 6.9Hz, 1.5Hz, 1H), 7.65 (d, *J*= 8.0Hz, 1H), 7.52 (ddd *J*=7.0Hz, 7.0Hz, 0.8Hz, 1H), 4.52 (s, 2H), 4. 22 (s, 2H) ppm. **^{13}C NMR** (150MHz, DMSO-d_6): δ_C172.14, 161.89, 154.11, 148.71, 134.93, 127.49, 127.12, 126.32, 121.90, 70.77, 68.43 ppm. **LC-MS** *m/z* calcd for $C_{11}H_{10}N_2O_4$ 234.21 [M+H$^+$]; 235.2 observed.

5-(4-oxo-3,4-dihydroquinazolin-2-yl)pentanoic acid- ME0817 (300a_09)
Synthesis: 2-Aminobenzamide (50.43 mg, 0.37mmol) and methyl adipoyl chloride (82.67mg, 0.4628mmol) were suspended in toluene (0.25M) and irradiated to microwave at 150 degree for 30 minutes. After completion of first step, NaOH (2M, 10eq) was added to the reaction mixture and irradiated to microwave at 100 °C for 30 minutes. The reaction mixture was monitored by TLC and LCMS. Then it was washed with NaOH (1M) and aqua layer was collected, acidified with HCl (6M) and extracted with EtOAc, dried over Na_2SO_4 and evaporated. The product was dried under vacuo as a yellow solid. The final product was purified using HPLC (A: aq. 0.75% HCOOH in H_2O, B: aq. 0.75% HCOOH in CH_3CN, organic phase gradient 25→100% over 25 min) to yield pure. **Chromatography:** A: aq. 0.75% HCOOH in H_2O, B: aq. 0.75% HCOOH in CH_3CN, organic phase gradient 25→100% over 25 min, white foam. **^{1}H NMR** (600MHz, DMSO-d_6): δ_H 12.18 (s, 1H), 8.08 (dd, *J*= 7.9Hz, 1.3Hz, 1H), 7.77 (ddd,*J*= 6.9Hz, 6.8Hz, 1.4Hz, 1H), 7.59 (d, *J*= 8.0Hz, 1H), 7.46 (ddd J= 7.1Hz, 7.1Hz, 0.7Hz, 1H), 2.60 (t, *J*= 7.5Hz, 2H), 2.25 (t, *J*= 7.3Hz, 2H), 1.74 (p, *J*= 7.6Hz, 2H), 1.56 (p, *J*= 7.5Hz, 2H) ppm. **^{13}C NMR** (150MHz, DMSO-d_6): δ_C 174.84, 162.32, 157.79, 149.36, 134.80, 127.27, 126.42, 126.17, 121.26, 116.56, 114.87, 34.64, 33.86, 26.77, 24.46 ppm. **LC-MS** *m/z* calcd for $C_{13}H_{14}N_2O_3$ 246.26 [M+H$^+$]; 247.3 observed.

2-((4-oxo-3,4-dihydroquinazolin-2-yl)methyl)benzoic acid- ME0818 (300a_12)
Synthesis: Methods B **Chromatography:** A: aq. 0.75% HCOOH in H_2O, B: aq. 0.75% HCOOH in CH_3CN, organic phase gradient 25→100% over 25 min, white foam. **^{1}H NMR** (600MHz, DMSO-d_6): δ_H 12.87 (s, 1H), 12.39 (s, 1H), 8.07 (dd, *J*= 7.9Hz, 0.8Hz, 1H), 7.89 (d, *J*= 7.6Hz, 1H), 7.70 (ddd, *J*= 6.9Hz, 7.0Hz, 1.3Hz 1H), 7.52 (ddd, *J*= 6.8Hz,6.5Hz, 0.8Hz, 1H), 7.443-7.368 (m, 4H), 4.33 (s, 2H) ppm. **^{13}C NMR** (150MHz, DMSO-d_6): δ_C 169.07, 162.19, 157.04, 149.27, 137.47, 134.68, 132.35, 131.58, 130.90, 129.89, 128.57, 127.53, 127.28, 126.45, 126.14, 121.29 ppm. **LC-MS** *m/z* calcd for $C_{16}H_{12}N_2O_3$ 280.28 [M+H$^+$]; 281.3 observed.

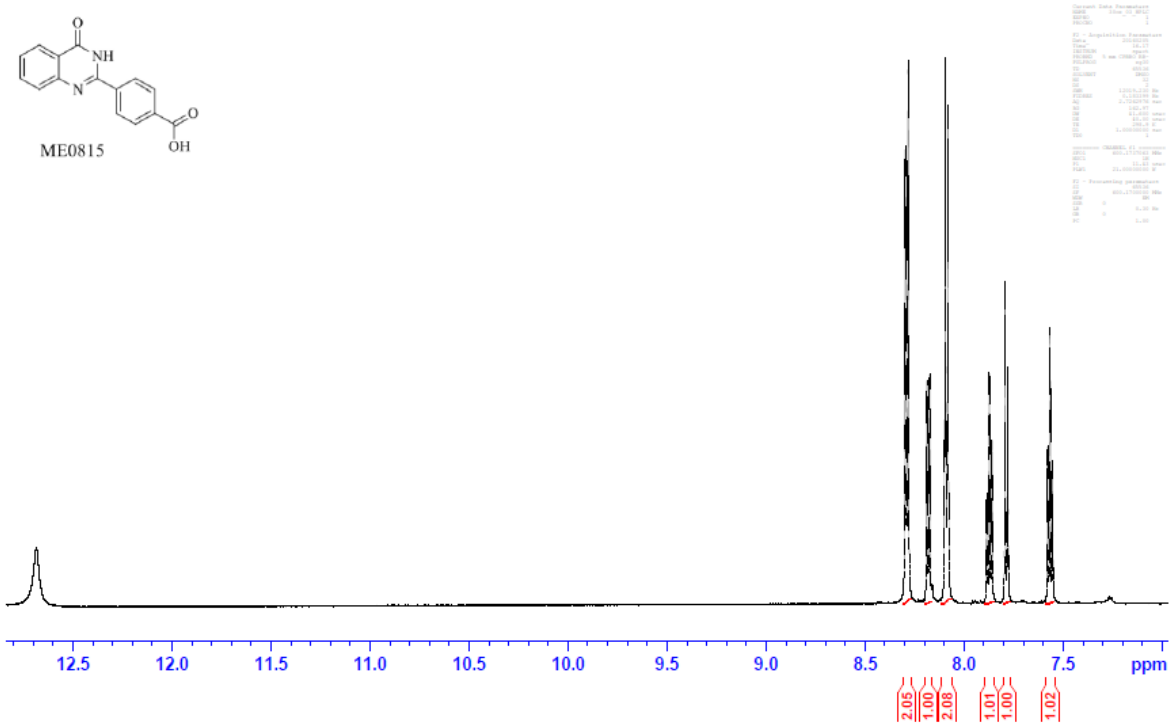
5-(4-oxo-3,4-dihydroquinazolin-2-yl)furan-2-carboxylic acid- ME0819 (300a_15)
Synthesis: Methods A **Chromatography** A: aq. 0.75% HCOOH in H_2O, B: aq. 0.75% HCOOH in CH_3CN, organic phase gradient 25→100% over 25 min. white foam. **^{1}H NMR** (600MHz, DMSO-d_6): δ_H 13.60 (s, 1H), 12.66 (s, 1H), 8.15-7.40 (m, 6H) ppm. **^{13}C NMR** (150MHz, DMSO-d_6): δ_C 161.97, 159.50, 148.84, 147.52, 143.90, 135.34, 128.09, 127.64, 126.45, 122.00, 119.37, 115.95 ppm. **LC-MS** *m/z* calcd for $C_{13}H_8N_2O_4$ 256.21 [M+H$^+$]; 257.2 observed.

2-(4-oxo-3,4-dihydroquinazolin-2-yl)benzoic acid- ME0821 (300a_37)
Synthesis: Methods B **Chromatography:** A: aq. 0.75% HCOOH in H_2O, B: aq. 0.75% HCOOH in CH_3CN, organic phase gradient 25→100% over 25 min, white foam. **^{1}H NMR** (600MHz, DMSO-d_6): δ_H 13.01 (s, 1H), 12.74 (s, 1H), 8.16 (dd, *J*= 7.9Hz, 1.278Hz, 1H), 7.95 (d, *J*= 7.5Hz, 1H), 7.82 (ddd, *J*= 8.3Hz, 1.5Hz, 1H), 7.70-7.63 (m, 4H), 7.53 (ddd, *J*= 7.9Hz, 0.9Hz, 1H) ppm. **^{13}C NMR** (150MHz, DMSO-d_6): δ_C 167.87, 162.12, 155.45, 149.37, 135.64, 134.77, 131.96, 131.61, 130.41, 130.28, 127.66, 126.87, 126.24, 121.57 ppm. **LC-MS** *m/z* calcd for $C_{15}H_{10}N_2O_3$ 266.25 [M+H$^+$]; 267.3 observed**.**

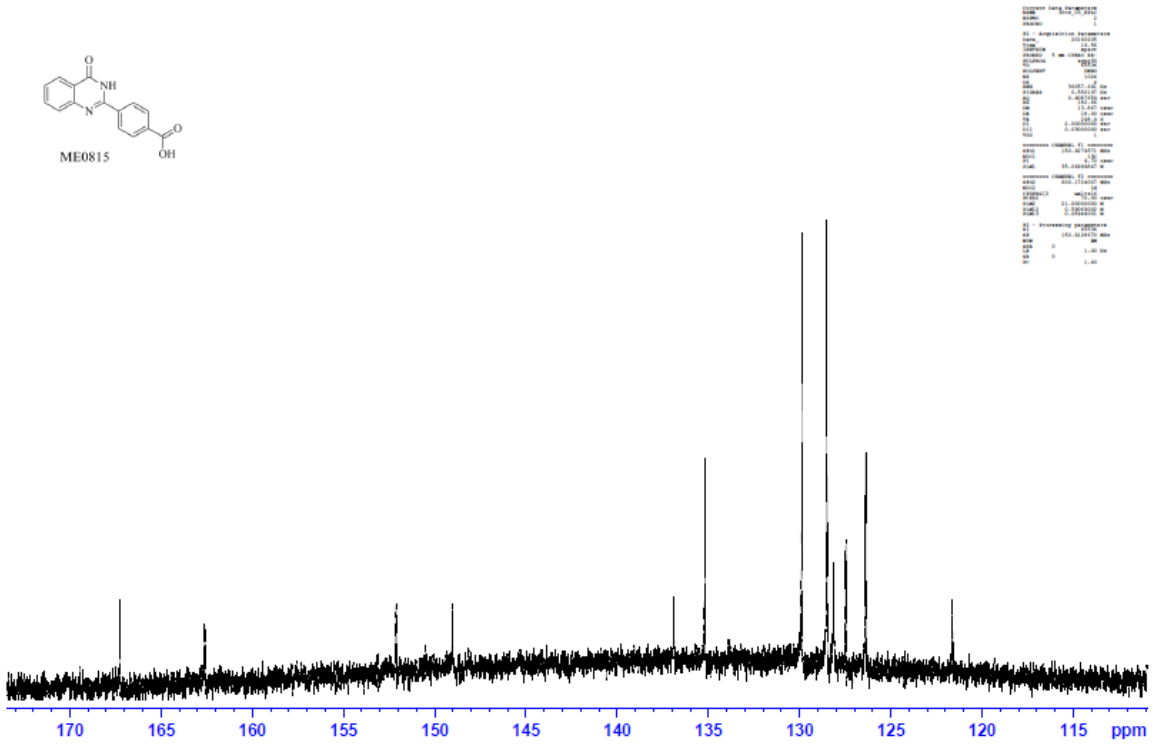
9. Appendix III

NMR data of **ME0815** and **ME0850**.

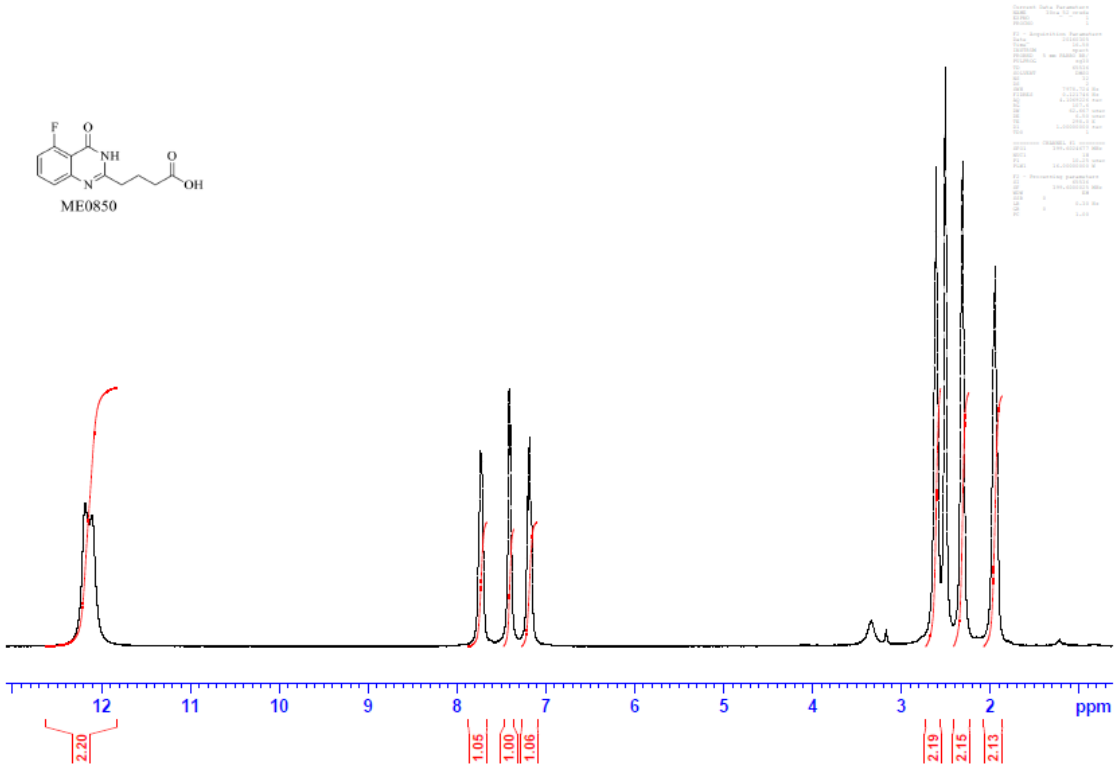
^{1}HNMR spectrum of **ME0815**



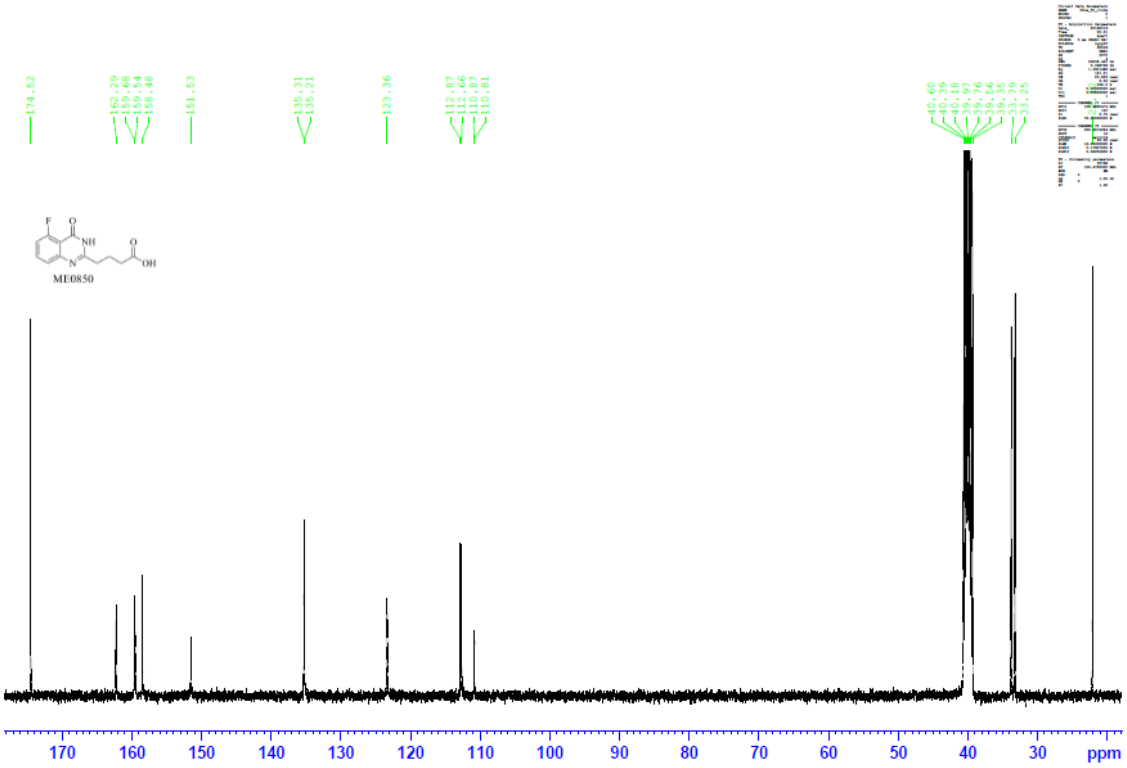
^{13}CNMR spectrum of **ME0815**



^{1}HNMR spectrum of **ME0850**



^{13}CNMR spectrum of **ME0850**



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