DISCOVERY OF SMALL MOLECULE INHIBITORS OF PROTEIN-PROTEIN INTERACTIONS

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Discovery of Small Molecule Inhibitors of Protein-Protein Interactions

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Protein-protein interactions (PPIs) constitute an emerging class of targets for the next generation of therapeutic intervention. Despite their fundamental role in many biological processes and diseases such as cancer, PPIs are still largely underrepresented in drug discovery. Although small molecule PPI inhibitors are highly valuable due to a number of advantages relative to biological agents in terms of production, delivery, titratability and cost, the robust discovery of lead compounds remains a great challenge. Two structure-based drug discovery strategies are described in this work to generate small molecules to target PPIs.

A receptor-based drug discovery approach can be applied when an accurate three-dimensional (3D) structure of a specific PPI complex is available. A novel, complementary and transformative approach for the rational design of small molecule inhibitors based on the crystal structure of the p53-Mdm2 complex was developed. This method is based on a tight interplay of structural biology information, the "anchor" concept, efficient chemical synthesis via multicomponent reactions (MCRs), as well as virtual and real screening processes. Applying the method we efficiently discovered several new scaffolds of inhibitors of the p53/Mdm2 interaction with lower micromolar affinity binding to Mdm2, which can serve as starting point for medicinal chemistry optimization. Advantages of our approach include high hit rates and less attrition based on the parallel discovery of multiple scaffolds, built-in optimization pathways using efficient MCRs, and fast generation of potential lead compounds. Potential anticancer drug

candidates were identified by biochemical assays, co-crystallization, cell based assays, as well as further preclinical evaluations (solubility, metabolism, pharmacokinetics, and xenograft studies).

A ligand-based drug discovery approach was explored since PPIs are critically dependent on "anchor" residues, which can serve as the pharmacophore model for small molecules. Multicomponent reactions were employed for design of novel scaffolds and DOS of drug-like compounds, since hit identification of PPI inhibitors via traditional approaches such as high throughput screening (HTS) is fundamentally limited by chemotypes present in the library collections. Novel and diverse scaffolds based on the privileged structures (1,4-benzodiazepines, 1,4-thienodiazepines) and "anchor" residues, which can be accessible from multicomponent reactions, were designed and synthesized. Compared with conventional methods, these approaches are advantageous to generate small molecules targeting PPIs in terms of efficiency, diversity, and economy.

In summary, the approaches described in this dissertation constitute important contributions to the fields of medicinal chemistry and structure-based drug discovery, which combine structural insights and ligand design to expedite the discovery of novel small molecule inhibitors of PPIs.

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LIST OF ABBREVIATIONS

antagonist-induced dissociation assay (AIDA)

1,4-benzodiazepin-2,5-dione (BDZ)

binding efficiency index (BEI)

diversity-oriented synthesis (DOS)

fragment-based drug design (FBDD)

fluorescence polarization (FP)

Gewald three-component reaction (G-3CR)

50% growth inhibitory concentration (GI_{50})

heteronuclear single quantum coherence (HSQC)

high throughput screening (HTS)

50% inhibition concentration (IC_{50})

inhibition constant (K_i)

isocyanide-based multicomponent reaction (IMCR)

multicomponent reaction (MCR)

murine double minute 2 (Mdm2)

National Cancer Institute (NCI)

Passerini three-component reaction (P-3CR)

Protein Data Bank (PDB)

root-mean-square deviation (RMSD)

solvent accessible surface area (SASA)

structure-based drug design (SBDD)

supercritical fluid chromatography (SFC)

1,4-thienodiazepine-2,5-dione (TDZ)

transcription-activation domain (TAD)

total polar surface area (TPSA)

1,5,7-triazabicyclo[4,4,0]dec-5-ene (TBD)

Ugi-5-center-4-component reaction (U-5C-4CR)

Ugi-deprotection-cyclization (UDC)

Ugi four-component reaction (Ugi-4CR)

PREFACE

I would like to take a moment to acknowledge those people who have contributed to my success in completing this dissertation.

First and foremost, I would like to express my gratitude to my research advisor and mentor, Professor Alexander Doemling, who has given me the opportunity to grow into an independent scientist. None of this work would have been possible without his support and guidance. I am tremendously grateful for my colleagues (Kareem Khoury, Dr. Wei Wang, Dr. Kan Wang, Dr. Barbara Beck, Dr. Haixia Liu, Dr. Haiping Cao, Dr. Tadamichi Nagashima) and undergraduate students (Dabin Kim, Tyler Chanas, Carly Sacco, James Gaugler, Valerie Nolt, Neal McCall, Yeong Han) in Dr. Doemling's group. I really appreciate them for many nice help along the way.

I had the good fortune to work with a number of excellent collaborators. The research described in this dissertation would not have been possible without contributions from them. I would like to thank Dr. Holak and his research group in Max Planck Institute of Biochemistry, Germany. I especially appreciate the long term collaboration with Siglinde Wolf. I also would like to thank Dr. Camacho, Dr. Koes and Dr. Meireles in Department of Computational and Systems Biology for collaborations. I wish to thank Dr. Eiseman and Dr. Beumer in University of Pittsburgh Cancer Institute for their contributions in preclinical studies.

1.0   INTRODUCTION

1.1   PROTEIN-PROTEIN INTERACTIONS AS DRUG TARGETS

Protein-protein interactions (PPIs) occur when two or more proteins (or subunits) bind together to form non-covalently bound complexes. More than 20,000 human PPIs have been deposited in protein interaction databases to date.[1] A wide variety of biological events are organized by PPIs, such as signal transduction, DNA replication, apoptosis, immunology, etc.[2] Many human diseases are the result of abnormal PPIs that involve endogenous and/or pathogen proteins.[3] PPIs represent prospective targets for human therapeutics that address unmet medical needs, such as cancer, diabetes and Alzheimer's disease.[4, 5] Therefore, discovery of potential therapeutic agents that modulate PPIs is an attractive and important research area.[6]

Targeting PPIs with small molecules is a valuable strategy in the development of new therapeutic agents. Therapeutic development of modulators of PPIs has, however, so far proven difficult. To date, there are very few examples of drugs in the market and drug candidates in clinical studies that function through targeting PPIs.[7] Small molecule modulators of PPIs can either stabilize or disrupt a given protein complex (**Table 1**).[8] For example, taxanes and epothilones bind to the β-subunit of the tubulin heterodimer and stabilize the interactions between tubulin heterodimers, ultimately leading to apoptosis of cancer cells.[9] On the other hand,

vinca alkaloids (vincristine and vinblastine) bind at the growing end of tubulin heterodimer and destabilize microtubule formation by disrupting tubulin-tubulin binding and ologomerization.[10]

Table 1. Small molecule modulators of protein-protein interactions

Stabilizers		Inhibitors	
	FK506 Calcineurin A/Calcineurin B (PDB: 1TCO)		**Nutlin-2** p53/Mdm2 (PDB: 1RV1)
	Rapamycin FKBP12/FRB-FRAP (PDB: 1FAP)		**IIA6B17** Myc/Max (PDB: 1NKP)
	Brefeldin A ARF1/ARF-GEF (PDB: 1R8Q)		**ABT-737** BAK/Bcl-X_L (PDB: 2YXJ)
	Fusicoccin A 14-3-3c/PMA2-CT52 (PDB: 2O98)		**SPD304** TNF-alpha (PDB: 2AZ5)
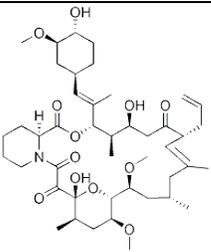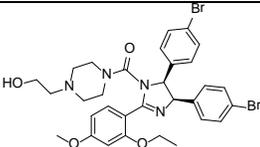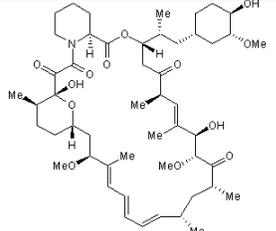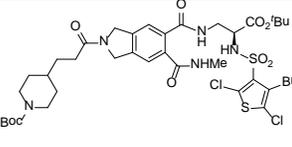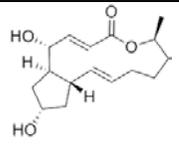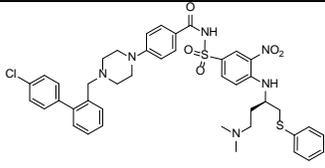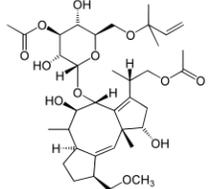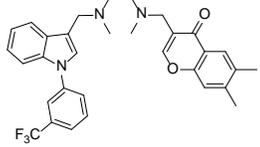
It is difficult to identify small molecule modulators of PPIs because of the overall characteristics of the interface.[11] PPIs typically involve a relative large surface area: i) standard-size interfaces have 1200-2000 Å^2; ii) the few smaller interfaces with 1150-1200 Å^2 normally constitute short-lived and low-stability complexes; iii) large interfaces in the range 2000-4660 Å^2 occur mostly between proteases and a particular class of inhibitors and between

G-proteins and other components of the signal transduction system; iv) the vast majority of protein heterodimer interfaces are larger than 600 Å^2.[12] The amount of buried surface area of protein-protein complexes greatly exceeds the binding area of small molecules. In many cases, the inherent flexibility and plasticity of the interface imposed by the structural and dynamical properties of PPIs makes it challenging to design modulators or binding site mimetics.[13] In addition, the interfaces of PPIs are often relatively flat and lack deep cavities (such as substrate binding sites of enzymes).[14] It may be difficult for small molecules to bind a shallow protein surface with high binding affinities.

1.2   DISCOVERY OF SMALL MOLECULE INHIBITORS

Unlike the traditional drug targets, PPIs remain challenging in terms of drug discovery. The development of competitive inhibitors provides the most direct approach to disrupting PPIs. Antibodies, recombinant proteins, and peptides have shown clinical success as inhibitors of extracellular PPIs.[15-19] Regarding the multitude of potential advantages of small molecules over biologics (such as oral bioavailability, stability, easy and efficient production, non-immunogenicity, to name just a few), there is an emerging need for the study of small molecule inhibitors to target PPIs. In addition, small molecules have demonstrated the value of targeting intracellular PPIs.[20] The feasibility of developing small molecules to block PPIs has been demonstrated.[21]

1.2.1 Screening of compound libraries

Traditionally, discovery of small molecule inhibitors relies on screening of compound libraries, such as collections of synthetic compounds, natural products, and combinatorial mixtures prepared by parallel synthesis methods. The advent of high throughput screening (HTS) enables the screening of thousands up to millions of samples in *in vitro* assays.[22] HTS is especially useful when there is a lack of knowledge about the function and structure of the biological targets, such as PPIs.[23] HTS still, however, suffers from a low success rate due to the false positives, promiscuous hitters, and low compound collection diversity. Hence, approaches have been applied to compensate for the accuracy and efficiency of finding active compounds, such as high-content screening and screening of focused compound libraries.[24, 25]

For example, a variety of chemotypes of p53/Mdm2(Mdm4) inhibitors were discovered via different means of screening technology (**Table 2**). HTS of large chemical libraries was used to discover the p53-Mdm2 inhibitors TDP222669 and SL-01, and the p53-Mdmx inhibitor SJ-172550. A high-content screening campaign of 220,000 compounds identified SID 17433115 as a p53-Mdm2 inhibitor.[26] Nutlins (*cis*-imidazoline-based lead compounds) were discovered by experimental screening of a diverse library of synthetic compounds, and Nutlin-3a was identified as first potent p53-Mdm2 inhibitor. Screening of focused compound libraries (natural products or synthetic compounds) was used to identify inhibitors of p53-Mdm2 interaction. Terphenyl 14, chalcone B and dehydroaltenusin were identified as p53-Mdm2 inhibitors via screening of a small number of compound collections.

Table 2. Screening hits of small molecule inhibitors of p53/Mdm2(Mdm4) interaction

High-throughput screening		Screening of focused compound libraries	
TDP222669	ThermoFluor (338,000 compounds screened)[27]	Nutlin-3a	Surface plasmon resonance assay (a diverse library screened)[28]
SID 17433115	Positional biosensor assay (220,017 compounds screened)[26]	Terphenyl 14	FP assay (21 terphenyl derivatives screened)[29]
SL-01	Mammalian two-hybrid assay (3,840 compounds screened)[30]	Dehydroaltenusin	T7 phage display assay (70 natural products screened)[31]
SJ-172550	FP assay (295,848 compounds screened)[32]	Chalcone B	ELISA-based assay (16 chalcones)[33]
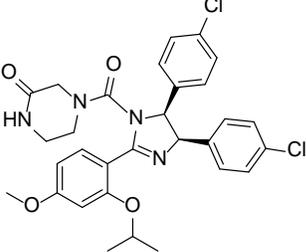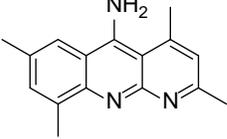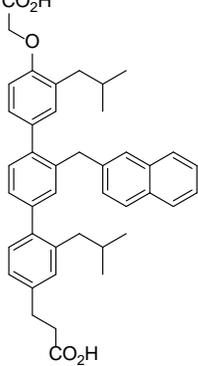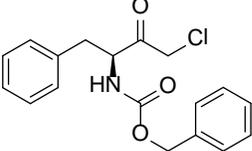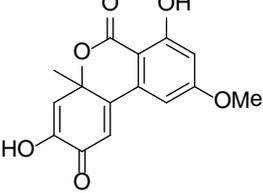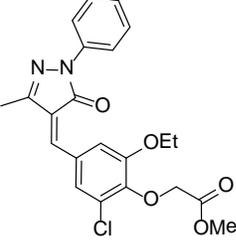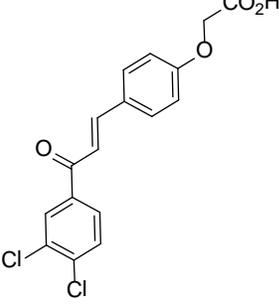
1.2.2 Fragment-based drug design

Fragment-based drug design (FBDD) is a relatively new drug discovery paradigm that has been successfully applied to generate small molecule inhibitors of PPIs.[34, 35] FBDD relies on screening of molecular fragments (MW < 300 Da) for binding activity against a target of interest. Subsequently, binding fragments are elaborated onto high-affinity compounds by growing a fragment (i.e., fragment evolution), linking two fragments, or merging fragments based on a common substructure.[36] Screening molecular fragments often results in higher hit rates than conventional HTS.[37] In terms of ligand efficiency, fragments often bind with high efficiency, with most of their atoms directly engaging in strong interactions with the target binding site.[38]

For example, the FBDD strategy has been successfully applied to discover drug candidates that inhibit the interaction between pro-survival Bcl-2 proteins and BH3 ligands. Abbott carried out a large fragment screening using 2D-NMR to discover fragments that bind to the hydrophobic BH3-binding groove of Bcl-x_L.[39] Two fragment hits, ABT-1 and ABT-2 (**Scheme 1**), were identified and shown to bind to distinct but proximal sites. NMR-based structural studies guided the linking of the fragments as well as subsequent optimization, which led to compound ABT-3. The subsequent modification using structure-based iterative library synthesis produced a lead compound ABT-4, which, however, was strongly deactivated in the presence of serum.[40] Further optimization resulted in ABT-737 with potent affinity (Kd < 1 nM) to Bcl-x_L, Bcl-2 and Bcl-w proteins.[41] Finally, a promising drug candidate ABT-263, which is orally bioavailable with 20-fold improvement in the pharmacokinetic/pharmacodynamic relationship, was identified.[42] Currently, ABT-263 is undergoing phase II clinical trials for the treatment of small-cell lung cancer and other malignancies.[43]
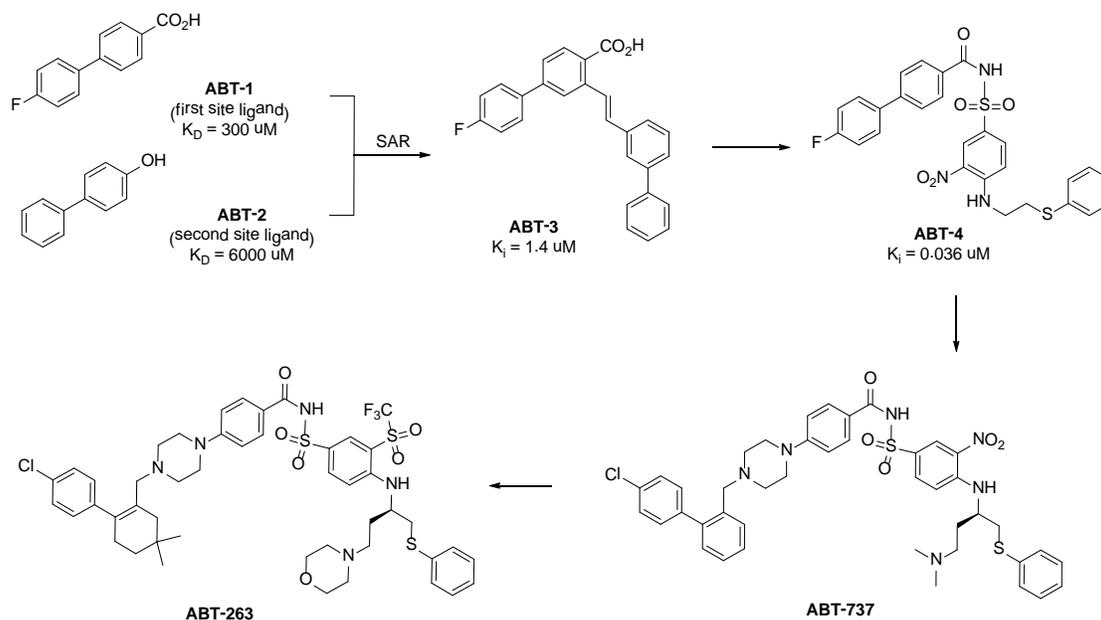
Scheme 1. From fragments to drug candidate: discovery of ABT-263

Wells and coworkers developed a more specialized approach to fragment screening, called tethering, which uses disulphide-bond formation to capture fragments that bind to a site of interest.[44] The tethering method of fragment discovery explores the reversible covalent bond formation, through thiol-disulfide exchange, between a cysteine residue near the binding site and disulfide-containing fragments.[45] Tethering has been particularly valuable in fragment discovery when applied to challenging targets, such as PPIs.[46] Using the site-directed fragment discovery method and fragment assembly, Ro26-4550 was identified as a small molecule inhibitor of a cytokine/receptor interaction (IL-2/IL2Rα).[35, 47]

1.2.3   Structure-based drug design

Although it is very challenging to discover small molecule inhibitors of PPIs, structure-based drug design (SBDD) approaches take advantage of three-dimensional structure information of

proteins and/or ligands to design novel drugs.[48] The power of SBDD has been demonstrated in the design of drugs targeting HIV-1 protease and neuraminidase, where structural information of protein targets has facilitated the discovery of several inhibitors as marketed drugs.[49, 50] The advancements in X-ray, NMR and other biophysical techniques for accurate structure determination have provided an increasing number of PPIs as potential "druggable" targets. When structural information of PPIs is available, computational methods can be used for rational design or screening of small molecule inhibitors.[48, 51] The availability of such information has encouraged *de novo* ligand design, where molecules can be designed using docking or other computer aided drug design to fit a binding site of protein-protein interaction.

Virtual screening (or in silico screening) is the most prominent example of SBDD to generate hit compounds by ranking large databases of chemical compounds.[52] Virtual screening approaches have emerged in drug discovery to identify lead compounds in a reliable and inexpensive manner.[53] Virtual screening involves computational modeling of ligand-protein complexes, and ranking according to defined scoring functions (i.e., minimized energy, shape and chemical complementarities).[54] Although they generally suffer from inaccurate treatment of solvation, flexible protein structure, and entropic effects, virtual screening methods continue to improve and have already demonstrated practical utility in many drug discovery campaigns targeting PPIs.[55-57]

A successful example of virtual screening for PPIs is the identification of NSC 66811, a cell-permeable, non-peptidyl, quinolinol compound that disrupts p53-Mdm2 interaction in the LNCaP prostate cancer cell line (**Figure 1**).[58] The virtual screening was performed on a subset (~150,000 compounds) of the National Cancer Institute (NCI) 3D database. From this database, 110,000 drug-like compounds were filtered and further screened using a pharmacophore model

derived from the crystal structure of Mdm2 complexed with the p53 peptide and several known nonpeptide small-molecule inhibitors. Hit compounds (2,599) were further scored by structure-based screening to dock each hit to the p53-binding site in Mdm2 and to rank their binding affinities. A total of 67 compounds from top ranking compounds were tested, and 10 hits defined by a Ki value of less than 10 µM were discovered. NSC 66811 had the highest binding affinity for Mdm2 (Ki = 120 nM).
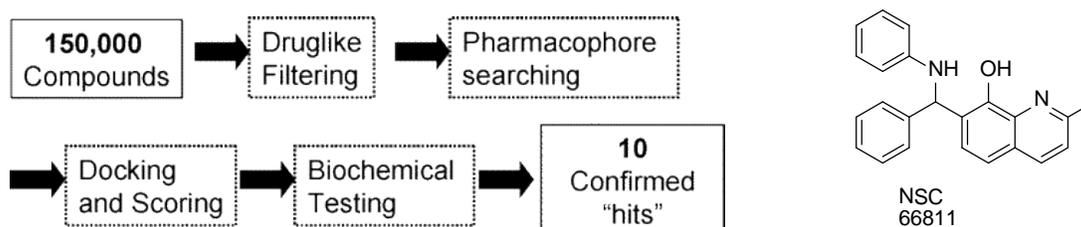
Figure 1. Virtual screening strategy for the discovery of NSC 66811[58] (Adapted with permission from American Chemical Society)

1.3   CURRENT RESEARCH

Design of drug-like, low-molecular weight compounds that can effectively inhibit PPIs has attracted the attention from both pharmaceutical industry and academia. The relative large interface between PPIs typically, however, exceeds the binding area for small molecules. Fortunately, certain protein-protein interfaces contain compact regions of amino acid residues that are crucial for the interaction.[59] These regions, called "hot spots", usually account for the major contribution of the free energy of protein-protein binding. Hotspot residues are usually identified experimentally as those residues that lead to a significant loss of binding affinity ($\Delta\Delta G$ > 1.5 kcal/mol) when mutated to alanine. Therefore, small molecules that mimic the amino acid

residues of "hot spots" may achieve high affinities with protein-protein binding interface in order to block the interaction.[60]

The PPIs that have a well-defined binding cavity and involve deeply buried "anchors" are potential targets for small molecule inhibitors.[61] Although endogenous small molecule ligands to bind at the protein-protein interaction are uncommon, these "anchors" can serve as starting points for the design of small molecule inhibitors. ANCHOR (http://structure.pitt.edu/anchor/) is a web-based tool to facilitate the analysis of protein-protein interfaces with regard to its suitability for small molecule drug design (**Figure 2**).[62] For a given protein-protein complex deposited in Protein Data Bank (PDB), ANCHOR calculates the change in solvent accessible surface area (ΔSASA) upon binding for each side-chain, along with an estimate of its contribution to the binding free energy.[63]

Figure 2. ANCHOR database[62] (Adapted with permission from Oxford University Press: 2770971385014)

ANCHOR exploits the so-called anchor residues (i.e., amino acid side-chains deeply buried at protein-protein interfaces), to indicate possible druggable pockets to be targeted by small molecules. Burriedness of amino acid side chains in a receptor protein is indicative for their role in a protein-protein interaction. In contrast to enzyme inhibitors, PPI inhibitors tend to

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occupy simultaneously several average-sized pockets.[64] Intuitively, the larger the number of anchors and their associated ΔSASA, the more "druggable" is the protein interface with a molecular weight limit for small molecular weight inhibitors. Bioisostere analogues of hotspot residues are ideal fragments to incorporate into compounds that might interfere with PPIs by mimicking the interactions encountered on the protein complex.

As described in this dissertation, two structure-based drug discovery strategies were used to generate small molecules to target PPIs. **Chapter 2** focuses on receptor-based approaches used to discover novel small molecule inhibitors of p53-Mdm2 interaction. Due to the high value and detailed structural characterization, a new SBDD approach was developed to generate novel p53-Mdm2 inhibitors. **Chapter 3** focuses on ligand-based approaches to generate diverse scaffolds and "anchor" biased compound libraries. Novel peptidomimetic scaffolds of small molecules were generated, and hit compounds were identified via screening against protein-protein interaction targets. These approaches aim to accelerate the challenge process for developing small molecule inhibitors of PPIs.

2.0   DISCOVERY OF SMALL MOLECULE INHIBITORS OF P53-MDM2 INTERACTION

2.1   INTRODUCTION

The tumor suppressor p53 protein, "the guardian of the genome", has an overarching role in protecting organisms from cancer.[65] As a transcription factor, human p53 is 393 amino acids long (43.7 kDa) and includes an N-terminus transcription-activation domain (TAD), which is important for apoptotic activity (**Figure 3**).[66] p53 is present at a very low cellular concentration in normal cells, while p53 accumulates in cells and becomes activated under certain conditions, such as hypoxia, DNA damage, or oncogene activation. p53 then binds to DNA and induces the transcription of downsteam genes (i.e., p21), which initiate cell cycle arrest, DNA repair, and apopotosis to prevent the proliferation of the damaged cells.[66] Loss of the p53 tumor suppressor pathway contributes to the development of most human cancers.[67] Restoration of the function of tumor suppressor p53 (such as Gendicine®) results in considerable therapeutic responses of tumors in cancer patients.[68]

Mdm2 (murine double minute 2) protein functions both as an E3 ubiquitin ligase that recognizes the N-terminal TAD of the p53 tumor suppressor,[69] and an inhibitor of p53 transcriptional activation (**Figure 3**).[70] Mdm2 acts as an oncogene in tissue culture systems, and the oncogenic potential of Mdm2 has been demonstrated.[71] Mdm2 (the human homolog is also called Hdm2) has been found amplified in more than 10% of 8000 human cancers from various

sites, including lung or stomach.[72] Mdm4 (the human homolog is also known as Mdmx, Hdm4 or Hdmx) was later identified as a p53-binding protein sharing structural homology with Mdm2.[73] Mdm4 has been found amplified or overexpressed in 10-20% of over 800 diverse tumors including lung, colon, stomach, and breast cancers, and 65% of retinoblastomas.[72]

p53 architecture
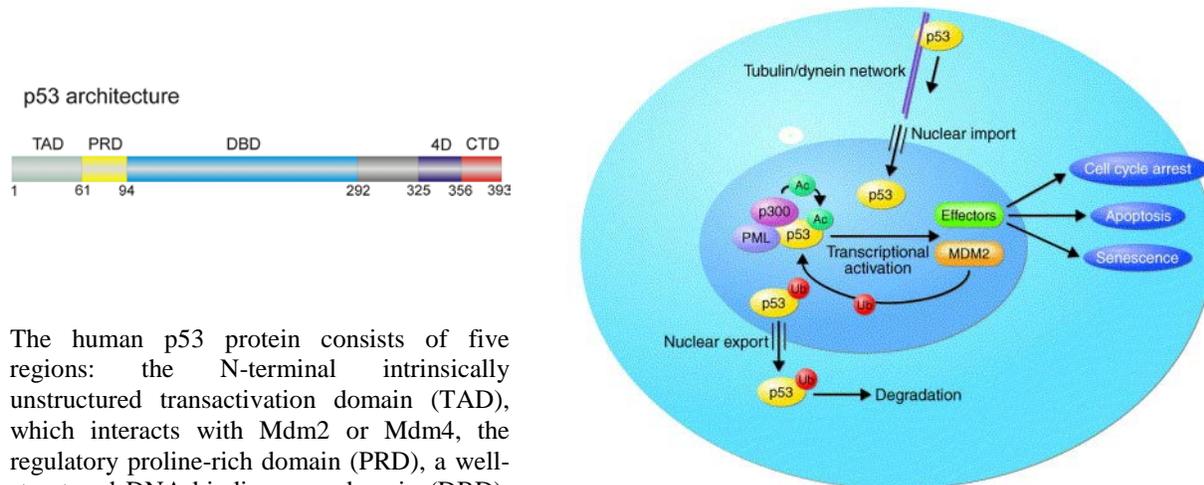
The human p53 protein consists of five regions: the N-terminal intrinsically unstructured transactivation domain (TAD), which interacts with Mdm2 or Mdm4, the regulatory proline-rich domain (PRD), a well-structured DNA binding core domain (DBD), the tetramerization domain (4D) and the C-terminal region.

A model depicting some of the mechanisms that may regulate p53 subcellular localization, stability and transcriptional activity. (Adapted with permission from Elsevier: 2770980154366)

Figure 3. Structure and function of p53[67]

Overall, Mdm2 (491 amino acids) and Mdm4 (490 amino acids) exhibit 32.6% amino acid sequence identity.[74] The N-terminal domain of Mdm4 shows the highest sequence similarity with the related domain of Mdm2 (53.6% identity). The BoxI BD is the most conserved domain, and a sequence comparison of amino acids most important for interaction with p53 are shown (**Figure 4**), with residues in bold that constitute the p53-binding hydrophobic pocket.[75] As shown in the co-crystal structures, the interactions between p53 and both its transcriptional inhibitors Mdm2 and Mdm4 are very conserved (**Figure 4**). On the other hand, the differential elucidation of the biological functions of both proteins are awaited with great interest.[76]

In view of the strong growth suppressive and pro-apopototic function of p53, the oncoprotein Mdm2 (and/or Mdm4) inhibits the function of wild type p53 in about 50% of human cancers.[77] The overexpression of the oncogene product Mdm2 (or Mdm4) is often observed in cancer cells, which negatively regulates the activity of wild-type p53. The relevance of Mdm2 (or Mdm4) on the regulation of p53 levels and activity has fostered the development of strategies aimed at restoring p53 functions.[78] A number of different small molecules and peptidomimetics disclosed in the last decade have been shown to block the physical interaction between p53 and Mdm2.[79] On the other hand, small molecule inhibitors of Mdm2 ubiquitin ligase activity have also shown the ability to stabilize and activate p53 in tumors that retain wild-type p53.[80]
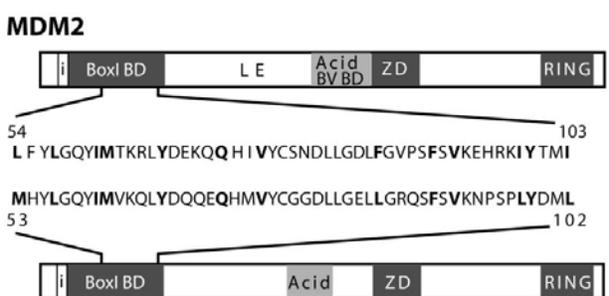
Comparison of Mdm2 and Mdm4 primary structures. The p53-BoxI binding domain (BoxI BD; amino acids ca. 25-110), the Zinc finger domain (ZD; aa ca. 290-330) and the RING domain (RING; aa ca. 435-482) are conserved. The rest sequence is not conserved.[75] (Adapted with permission from Elsevier: 2770990646612)
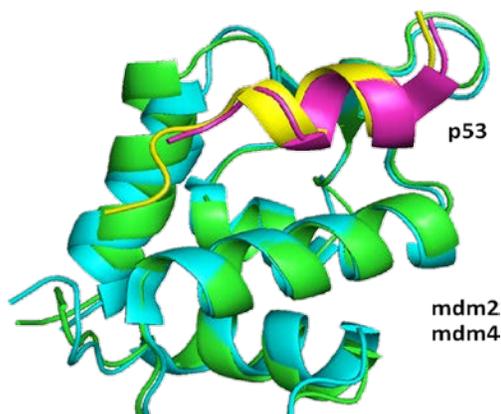
Strucutres of p53-Mdm2 complex (PDB: 1YCR; p53-yellow, Mdm2-green) and p53-Mdm4 complex (PDB: 3DAB; p53-purple, Mdm2-blue).

Figure 4. The interaction between p53 and Mdm2 (Mdm4)

Targeted manipulation of apoptosis in tumor cells provides a strategy for the rational design and discovery of novel anticancer agents. The PPI site between the transcription factor p53 and its negative regulator Mdm2 is a major target in current cancer drug discovery.[81] Disrupting the interaction between p53 and Mdm2 was shown to restore the wild type p53 activity and drive cancer cells selectively into apoptosis.[81] Many investigations of small

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molecule p53-Mdm2 inhibitors in different cancer cell lines and animal models support their usefulness as potential anticancer agents with a novel mode of action.[82] In fact, recent interim results from the first-in-class clinical p53-Mdm2 inhibitor RG7112 (a nutlin-3 derivative) in patients with relapsed/refractory acute myeloid and lymphoid leukemia and refractory chronic lymphocytic leukemia/small cell lymphocytic lymphomas are encouraging.[83] While several classes of small molecule p53-Mdm2 inhibitors have been described in the past, only some are of sufficient potency and few have been characterized by co-crystal structure analysis.[28, 84-88] The interface between p53 and Mdm2 is, however, accessible to small molecule drug discovery due to its dimension, concavity and hydrophobicity of the binding site.[89] Current p53-Mdm2 inhibitors have been discovered by different techniques, including high throughput screening (HTS), computational HTS and structure-based design (**Table 3**).[27, 28, 86, 87]

Table 3. Inhibitors of p53-Mdm2 interaction

Ligand	Structure	Ki (μM)	MW (Da)	Ref.
p53 residues 15-29 (PDB: 1YCR)		0.6	1808	[90]
Stapled peptide (SAH-p53-8)		0.055	2180	[91]
Nutlin-2 (PDB: 1RV1)		0.09	581	[28]
TDP222669 (PDB: 1T4E)		0.067	566	[27]
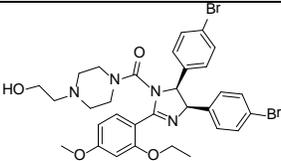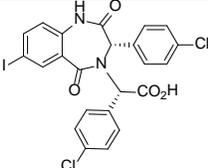
MI-63 analog (PDB: 3LBL)		0.036	577	[92]
Amgen 1a (PDB: 3JZK)		11	536	[93]
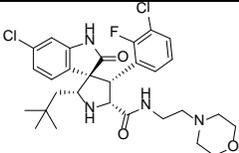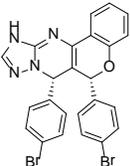
The visualization tool of the ANCHOR Database shows the interaction between Mdm2 (surface representation) and three anchor residues from p53 (stick representation) in **Figure 5**.[62] These three residues are among those with the largest ΔSASA and lowest (i.e. favorable) predicted binding interaction energy. Note that the table of interacting residues also shows the C-terminal Asn29 as having a large value of ΔSASA and unfavorable predicted energy (+3.7 kcal/mol) due to the extra carboxylic acid group. Thus, as is, Asn29 is predicted to be not a good group to target for drug design. On the other hand, the three selected residues (Phe19, Trp23 and Leu26) are indeed hotspot anchors that have been exploited on the design of compounds that inhibit the interaction between p53 and Mdm2.[94]
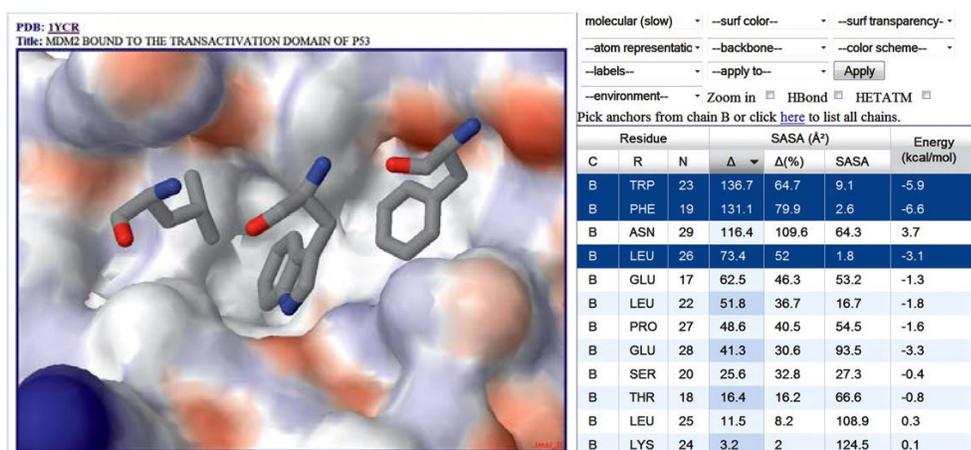
Figure 5. Visualization tool showing anchor residues Phe19, Trp23 and Leu26 of p53 (PDB: 1YCR)[62]

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2.2   STRUCTURE-BASED APPROACH FOR THE RATIONAL DESIGN OF P53-MDM2 INHIBITORS*

*Adapted with permission from John Wiley and Sons: 2761080520679

The discovery of a lead compound is an essential process in early drug discovery, hopefully eventually resulting into a clinical candidate and a drug for the treatment of a disease. Besides affinity and selectivity for the target, however, other target unrelated compound properties are equally important for the fate of drug candidate, e.g., water solubility, lipophilicity and molecular weight since they determine important aspects such as oral bioavailability, dosing schedule and side effects. The parallel discovery and early development of several leads is therefore now pursued whenever possible, an approach that takes into account the high attrition rate of early drug discovery projects. Currently, hits as starting points for medicinal chemistry optimizations are mostly found by HTS campaigns and to a much less extent by structure-based approaches including fragment-based and computational drug discovery. For certain target classes, however, HTS often yields very low numbers of hits.[57, 95] For example, PPIs are notoriously difficult to hit with drug-like small molecules.[11] This has been assigned to the unusual structure, topology and flexibility of protein-protein interfaces.[96] The recent advancement of several drugs into clinical development clearly shows that certain PPIs, e.g., Bcl-x and XIAP, can be efficiently targeted by small molecules.[97, 98] Thus, as described in this dissertation, a complementary process was developed that led to the parallel discovery of several compounds belonging to seven different scaffold classes, amenable to synthesis by efficient multicomponent reaction (MCR) chemistry in just one step, that antagonize the PPI between the transcription factor p53 and its negative regulator Mdm2.

PPIs are often mediated by only a few key amino acid side chains and the terms "hot spot" and "anchor" have been introduced for such locally constrained areas and amino acids on the surface of interacting proteins.[11, 99-101] In a first approximation, the depth into which a specific amino acid side chain of the donor protein is buried in the acceptor protein is indicative of its energetic importance. We reasoned that this "anchor" amino acid side chain might serve as reasonable starting point for the design of inhibitors of a PPI. Thus, we use this particular amino acid side chain as an initial "anchor" in virtual libraries of low molecular weight scaffolds (**Figure 6**). Virtual compounds containing anchor side chains are selected for the synthesis and screening based on docking into the protein-protein interaction interface. The starting pose for the docking/energy minimisation procedure is forced in such a way that an overlap between the anchor and the template amino acid side chain is ensured. In order to rapidly test these ideas, an efficient and fast but versatile synthesis approach, the MCR was chosen.[102-109] MCR allows the assembly of many diverse and complex scaffolds in a one-step/one-pot manner, thus saving time and resources, and potentially increasing the hit rate of drug discovery.
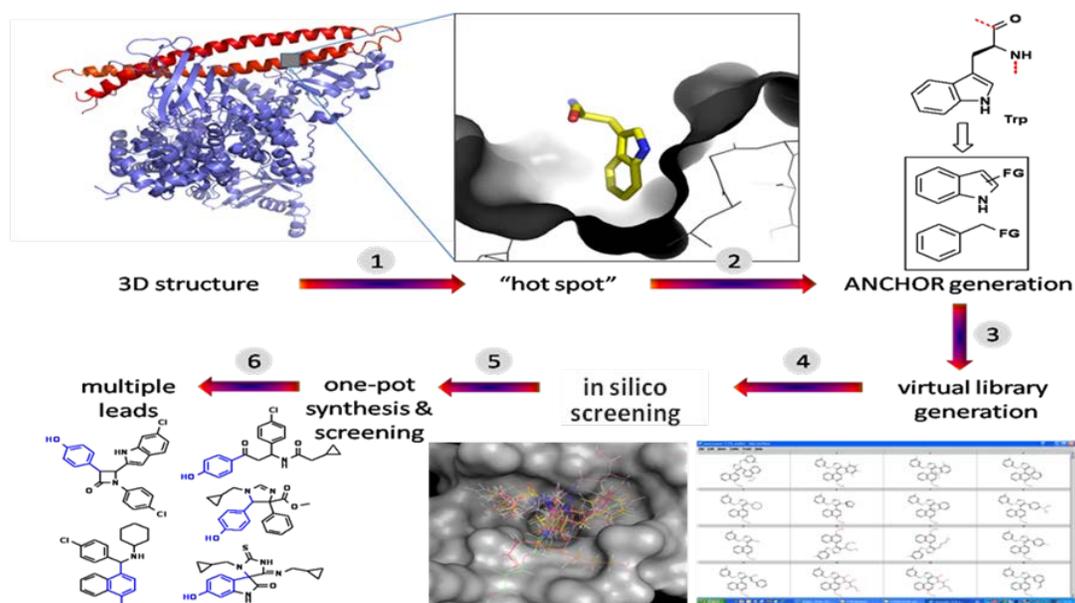
Figure 6. Graphical representation of the workflow in the discovery procedure for the rapid generation of small molecular weight inhibitors of protein-protein interactions. The process relies on high resolution structural target

data (1), the presence and identification of a highly buried amino acid (2), a fragmentation/anchor generation step (3), virtual chemistry employing the anchor and based on MCRs (4), constrained docking forcing the anchor fragment into the binding site (5), and synthesis and screening (6). (FG = functional group).

The PPI interface of p53-Mdm2 has been characterized in molecular detail by X-ray structure analysis.[90] It relies on the steric complementarity between the Mdm2 cleft and the hydrophobic face of the p53 α-helix and, in particular, on a triad of p53 amino acids Phe19, Trp23, and Leu26, which insert deep into the Mdm2 cleft (**Figure 7**). The indole side chain of Trp23 was chosen as the "anchor" residue in the approach for three reasons: 1) it is the central amino acid of the triad, thus facilitating addressing by the inhibitors the crucial Phe19 and Leu26 binding sites; 2) it is deeply buried in Mdm2; and 3) it also features, in addition to extensive van der Waals contacts, a hydrogen bond to the Leu54 backbone carbonyl of Mdm2. In fact, calculation of the solvent-accessible surface areas of all amino acids in the p53/Mdm2 interaction ranks Trp23>Phe19>Leu26 the highest.[101] Next, from the in-house database of several hundred MCR scaffolds, 40 MCR scaffolds to create virtual compound libraries were selected.[102-109] By design, each of the compounds from the different scaffolds incorporated the anchor. Indole and bioistosteric 4-chlorophenyl derivatives[27, 28] endued with the corresponding functional groups were used as anchors to act as starting materials for the MCRs.

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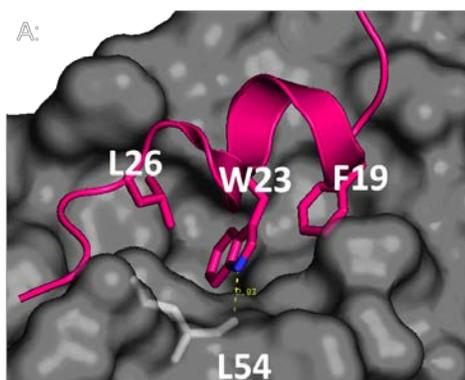
Figure 7. Architecture of the "anchors" in the p53/Mdm2 interface (PDB Identifier: 1YCR). The triad of amino acid side chains, Phe19, Trp23 and Leu26 of the interacting p53 α-helix make strong van der Waals contacts to the Mdm2 receptor. Trp23 additionally forms a hydrogen bond to Mdm2's Leu54 backbone carbonyl. The Mdm2 surface is shown in grey. The hot spot amino acid side chains of p53 are shown as pink sticks and the p53 α-helix in cartoon format.

The virtual library of compounds comprising all possible stereoisomers was generated using REACTOR software.[110] Different aliphatic and aromatic starting materials to represent different shape and electrostatic features were chosen for the MCR starting material classes. The virtual compound libraries incorporating the anchor side chain were docked into a rigid model of the p53 binding site in the Mdm2 receptor (PDB identifier 1YCR) using the modelling/docking software package Moloc.[90, 111, 112] Assuming that the anchor residue predefines the binding site of the molecules and in order to avoid non-productive docking poses, the anchor part (indole or 4-chlorophenyl) of the compounds was forced to overlap with the respective Trp23 anchor as a starting point for energy minimization of molecular models. As an encouraging evidence for the anchor approach, the Holak group previously solved the crystal structure of the MCR compound **PB12** bound to Mdm2 (**Figure 8**).[92] This structure shows an almost perfect alignment of the Trp23-indole anchor with the indole moiety of **PB12** with an root-mean-square deviation (RMSD) of 0.34 Å (indole).
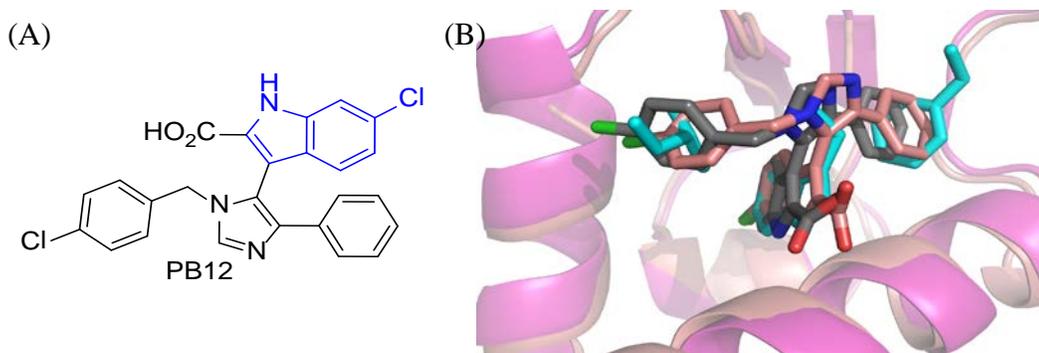
Figure 8. Cocrystal structure of **PB12**/Mdm2 complex. (A) The structure of PB12; (B) Alignment of the anchor p53 residues Phe19, Trp23 and Leu26 (cyan sticks) bound to Mdm2 (pink cartoon, PDB ID: 1YCR) with a van Leusen imidazole **PB12** based inhibitor (pink sticks) bound to Mdm2 (pink cartoon, PDB ID: 3LBK) and the corresponding predicted docking pose of **PB12** (grey sticks).
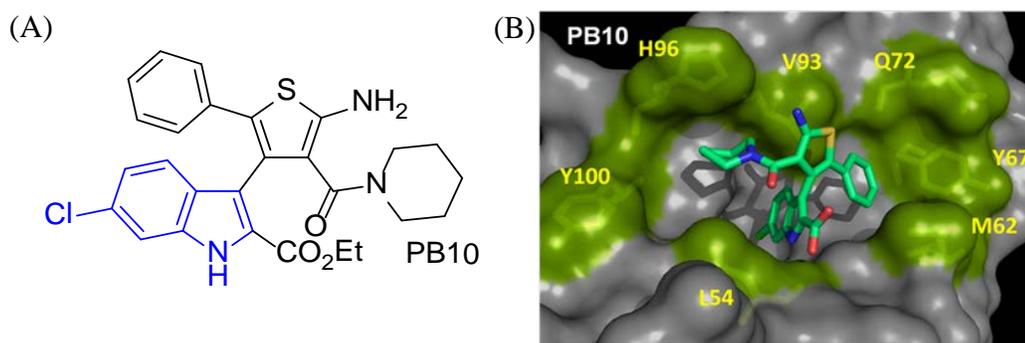
Figure 9. Binding model of **PB10** by constrained docking into p53 binding site. Several rim key amino acids of the receptor Mdm2 are shown as sticks and green surface and numbered.

A representative docking pose of one of the predicted p53/Mdm2 inhibitor compounds is shown in **Figure 9**. Next, compounds were screened for their ability to bind to Mdm2 and to antagonize the p53/Mdm2 PPI (**Figure 10**). NMR spectroscopy was used to test the compounds abilities to bind and antagonize the p53/Mdm2 complex as it can provide a wealth of information that otherwise cannot be assessed in high-throughput assays. Importantly, use of NMR-based screening allowed for determination of both compound affinity to Mdm2 and the ability of compounds to dissociate the preformed p53/Mdm2 complex. Other important questions about the compound properties can also be answered with an NMR-based approach. These include: Is the

compound sufficiently water soluble? Does the compound bind to Mdm2 or to p53? Does the compound cause precipitation of any of the proteins? Does the compound lead to major conformational changes in the binding protein? What is the binding site of the compound on the protein surface?
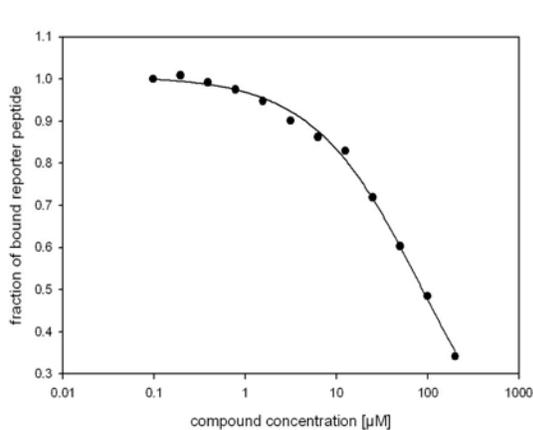
FP assay: Competitive displacement of the p53 FAM-P5 peptide from Mdm2 by compound **PB10.**
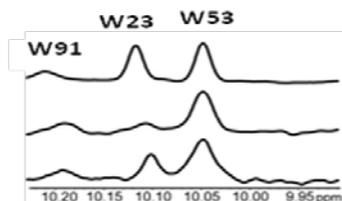
AIDA-NMR analysis: Upper trace: spectrum of p53 (residues 1-321). The three peaks are tryptophans of p53: Trp91, Trp23 and Trp53. Middle trace: the spectrum of the complex of p53 (res. 1-321) + Mdm2 (res. 1-125). Tryptophans 53 and 91 are not sensitive to the binding to Mdm2. Trp23 is in the binding site and therefore disappears on binding to Mdm2. Lower trace: addition of **PB10** to the complex releases p53 as seen by the reappearance of the tryptophan Trp23.

Figure 10. Fluorescent polarization (FP) assay and antagonist-induced dissociation assay (AIDA) analysis.

All of the information is instrumental in order to optimize a compound series not only for potency and selectivity but also for, e.g., water solubility and protein binding. Moreover, the usage of screening techniques based on independent physical methods prevents the discovery of PAIN compounds (Pan Assay Interference Compounds) and covalent binders.[113, 114] The different compounds were tested for binding to Mdm2 by performing a series of NMR titrations with isotopically enriched ^{15}N-Mdm2.[115, 116] Strong binding of a compound to its target is indicated by appearance of splitting of the signals in a heteronuclear single quantum coherence (HSQC) spectrum, whereas a shift of signals indicates weaker binding. Additionally we used our recently introduced antagonist-induced dissociation assay (AIDA), a fast 1D-NMR method to determine the K_D (**Appendix A**).[117, 118]

In summary, a new process for predicting and producing inhibitors of the cancer-relevant PPI between p53 and Mdm2 is introduced. The approach differs substantially from currently used techniques, including HTS, fragment-based approaches, structure-based design or computational screening of compound libraries. For example, classical fragment-based drug discovery approaches are strong in detecting weakly but efficiently binding small molecular units, which serve as starting points to assemble more potent drug-like compounds, however with no straightforward synthetic pathway.[119] Herein, the chemistry to more potent leads starting from the fragment (=anchor) is predefined by the MCR chemistry. Thus, a seamless pathway for the optimization of affinity and other important drug properties is predefined. Nutlin-3 is synthesized in a sequential > 8-step synthesis, while **PB10** is accessible in only two steps from commercial available starting materials.[120, 121] The identified hit compounds are drug-like and can be optimized regarding potency and physicochemical properties. Certainly, this new approach, successful in one target example, demands further validation with different types of PPIs.

2.3 UGI MULTICOMPONENT REACTION FOR THE DISCOVERY OF P53-MDM2 INHIBITORS

The examples shown in **Chapter 2.2** encouraged us to generate novel small molecule inhibitors of p53-Mdm2 interaction using the proposed structure-based drug discovery approach. Hence, a novel chemotype of p53-Mdm2 inhibitors was identified, which was accessible from the Ugi four-component reaction (Ugi-4CR). The structure-activity relationship study of the p53-Mdm2 inhibitors derived from the Ugi-4CR was extensively investigated in **Chapter 2.3.1**. The lead

compound was identified, which allows the futher optimization of protein binding in **Chapter 2.3.2**. Several potent p53-Mdm2 inhibitors were discovered by systematically varying the fluorine substitution pattern around a benzyl group that undergoes stacking interactions with His 96 of Mdm2.

2.3.1 Ugi multicomponent reaction derived p53-Mdm2 inhibitors

Herein, the use of an alternative technique, the web- and structure-based design tool ANCHOR.QUERY was described, which led to the discovery and the subsequent optimization of new, potent p53-Mdm2 inhibitors based on the Ugi MCR. The key biophysical insights of ANCHOR.QUERY are that anchor residues define a binding mode and the structural alignment of a chemical mimics the corresponding anchor residue in the PPI structure.[62] An anchor residue is defined as an amino acid side chain that is deeply buried in the protein-protein interaction interface. Another web server, ANCHOR.DataBase, is available to calculate the buriedness of the interface amino acid side chains.[62] In the case of the p53-Mdm2 interaction, Trp23 is the most deeply buried and central p53 amino acid, and was therefore selected as the anchor. The importance of this amino acid for the p53-Mdm2 interaction is also well documented by mutational studies.[122] Other deeply buried amino acid side chains of the p53 hot-spot, Phe19 and Leu26 were selected as hydrophobic pharmacophores.

Subsequently a ~1/2 billion conformer library based on ~5 million unique MCR compounds containing the indole anchor were aligned with the Trp23 anchor of p53 and screened for matching the anchor/pharmacophore model. The screening results were then sorted and ranked by molecular descriptors. For example, molecular weight ranking is important for the selection of the compounds to potentially achieve good ligand efficiency. The scaffold and

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individual compounds are chosen for synthesis according to the binding poses and the electrostatic complementarity of the molecules. This method is validated by several known MCR scaffolds as p53-Mdm2 inhibitors, including van Leusen 3-CR imidazole, Ugi-5C-4CR iminodicarboxylic acid amide, Ugi-4CR hydantoin, and Orru-3CR imidazolidine.[84, 85, 123-125] The proposed virtual molecule based on the Ugi-4CR with the lowest molecular weight and matching the pharmacophore points is shown in **Figure 11**. This serves as a starting point for validation and optimization in the discovery of new p53-Mdm2 inhibitors.

Figure 11. Screenshot of pharmacophore-based virtual screening platform ANCHOR.QUERY to discover MCR-derived Mdm2 inhibitors. The Mdm2 receptor is shown in surface representation (PDB ID: 1YCR). The hot spot p53 derived amino acids Phe19 and Leu26 are shown as lines and green spheres and the Trp23 indole anchor as yellow disk. The virtual U-4CR product (grey sticks) with the lowest molecular weight and matching with the pharmacophore model is depicted.

The Ugi-4CR is part of the ANCHOR.QUERY scaffolds due to its great scope and the reliability and compatibility of diverse inputs.[126, 127] For example, 3-formylindole derivatives that serve as ANCHOR building blocks have been previously employed in the U-4CR for the synthesis of alkaloid-like skeletons.[128, 129] A preliminary investigation by the Ugi reaction of 3-formylindole **1**, benzyl isocyanide, primary amine (isobutylamine or benzylamine, **Scheme 2**), and acetic acid gave the desired condensation products **7a-b** (room temperature, 2 days). The

corresponding deprotected products **8a-b** were obtained by the subsequent deprotection of **7a-b** using a 10% solution of TFA in dichloromethane (room temperature, overnight). A series of methods (Method A-C, **Scheme 2**) for the synthesis of **8c-t** were developed to account for the reactivity of diverse starting materials. Basic hydrolysis of the ethyl ester of the indole derivatives **8** produced the compounds **9** with a carboxyl group at 2-position (Method D, **Scheme 3**).
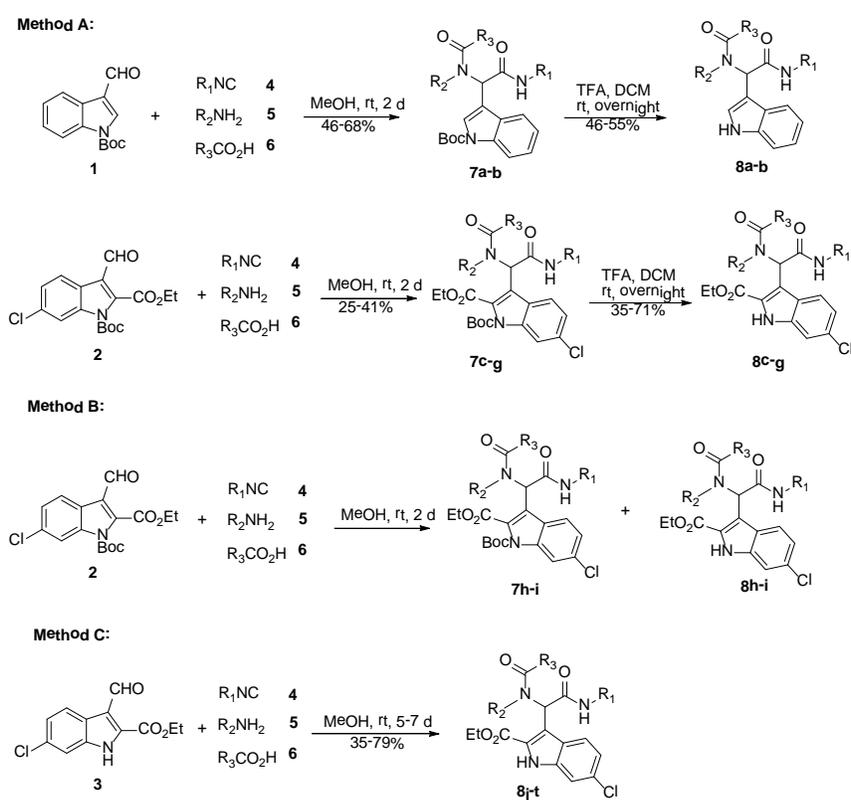
Scheme 2. Ugi-4CR of 3-formylindoles
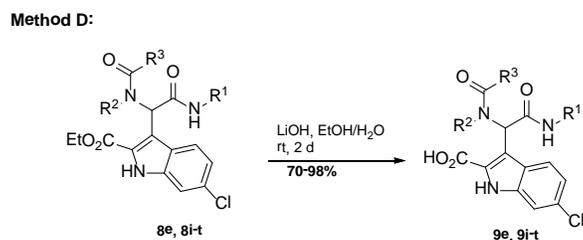
Scheme 3. Synthesis of indole-2-carboxylic acid derivatives **9**

A fluorescence polarization (FP) assay was employed to measure the binding affinities of small molecules with Mdm2 as previously described (Table S1).[124] As a reference compound, Nutlin-3a's Ki was found to be 0.04 µM, which is in very good agreement with previously reported value.[124] Additionally a complementary physically independent assay AIDA, was used to validate the potency of p53-Mdm2 inhibitor in an NMR competition binding experiment.[117, 118] In contrast to compound **8a** (AIDA: $K_D > 50$ µM), compound **8b** was identified as a hit with weak binding affinity to Mdm2 (AIDA: $K_D = 30$ µM). The compounds **8c-g** were synthesized using 3-formylindole **2**, since the introduction of 6-Cl to the indole fragment is known to increase the binding affinity with Mdm2.[130] For example, compound **8e** has low micromolar binding affinity to Mdm2 (AIDA: $K_D = 1.7$ µM). Encouraged by these results, compounds **8h-t** with different substitutions were synthesized and screened by the FP assay (**Table 4**). The binding is tolerant of the hydrophobic fragment introduced by several isocyanides (R^1: benzyl, cyclohexyl, *tert*-butyl), which is designed to occupy the Phe19 pocket of Mdm2. It turns out, however, that the binding is more sensitive to the hydrophobic fragment introduced by the amine (R^2), which is designed to occupy the Leu26 pocket of Mdm2. Compound **8l** was found as the most potent one in this series, which indicates the optimal combination of the fragments.

Table 4. SAR study of compounds **8a-t**
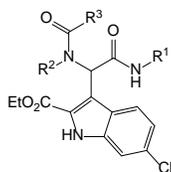
ID	R^1	R^2	R^3	Ki (µM)[1]
8a	benzyl		Me	n.i.
8b	benzyl		Me	n.i.

8c	benzyl	(isopropyl-CH₂)	Me	4
8d	benzyl	(phenyl-CH₂)	Me	6
8e	cyclohexyl	(phenyl-CH₂)	Me	2
8f	cyclohexyl	Cl—(phenyl)-CH₂	Me	22
8g	cyclohexyl	Cl, Cl—(phenyl)-CH₂	Me	9
8h	cyclohexyl	(phenyl-CH₂)	^{n}Pr	25
8i	cyclohexyl	(phenyl-CH₂)	H	14
8j	cyclohexyl	Cl—(phenyl)-CH₂	H	50
8k	cyclohexyl	F—(phenyl)-CH₂	H	30
8l	*tert*-butyl	Cl—(phenyl)-CH₂	H	1.8
8m	*tert*-butyl	Cl, Cl—(phenyl)-CH₂	H	4
8n	*tert*-butyl	Cl, Cl—(phenyl)-CH₂	H	10
8o	*tert*-butyl	Cl—(pyridyl)-CH₂	H	2.7
8p	*tert*-butyl	Cl—(phenyl)-CH(CH₃)	H	n.i.
8q	*tert*-butyl	Cl—(phenyl)-CH(CH₃)	H	8
8r	*tert*-butyl	HO—(phenyl)-CH₂	H	11
8s	*tert*-butyl	Ph—(phenyl)-CH₂	H	n.i.
8t	*tert*-butyl	PhO—(phenyl)-CH₂	H	n.i.

[1] Inhibition constant (K_i) was measured by FP assay. The abbreviation n.i. stands for "no interaction".

Compounds **9e-t** were synthesized by hydrolysis of the ethyl ester of the corresponding indole derivatives **8** and screened with the FP assay (**Table 5**). The binding affinities of **9e-t** improved overall compared with the corresponding parent ethyl ester compounds. It has previously been shown for the imidazole scaffold that the carboxylic acid group of the indole fragment contributes to the binding with Mdm2 (PDB ID: 3LBK).[92] The binding was also influenced by the fragment introduced by the acid component (R^3); small substitutents (H, Me) were well tolerated. Compound **9l** (K_i = 400 nM, AIDA: K_D = 300 nM) was found as the most potent in this series. Interestingly, none of the compounds showed comparable binding affinity with Mdm4 (data not shown), although Mdm4 shows significant overall sequence and very close shape similarity to the p53 binding site.

Table 5. SAR study of compounds **9e-t**
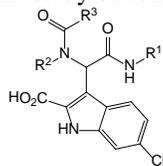
ID	R^1	R^2	R^3	K_i (µM)[1]
9e	cyclohexyl		Me	1.6
9i	cyclohexyl		H	1.6
9j	cyclohexyl	Cl—benzyl	Me	2.3
9k	cyclohexyl	F—benzyl	H	n.d.
(±)9l	*tert*-butyl	Cl—benzyl	H	0.4
(+)9l	*tert*-butyl	Cl—benzyl	H	0.3
(-)9l	*tert*-butyl	Cl—benzyl	H	0.7
9m	*tert*-butyl	Cl,Cl—benzyl	H	0.6

9n	*tert*-butyl		H	0.5
9o	*tert*-butyl		H	4
9p	*tert*-butyl		H	10.5
9q	*tert*-butyl		H	0.9
9r	*tert*-butyl		H	11
9s	*tert*-butyl		H	2.5
9t	*tert*-butyl		H	1.8
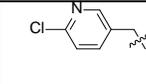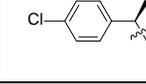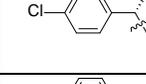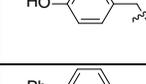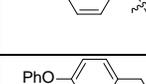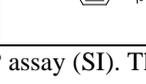
[1] Inhibition constant (Ki) was measured by FP assay (SI). The abbreviation n.d. stands for "not determined".

In order to indicate the role of the fragment introduced by the carboxylic acid (R^3) in protein binding, we further investigated the activity of compounds **11** (**Scheme 4**). The deformylation of the formamide produced by the Ugi reactions were observed under acidic conditions leading to the free amine.[131, 132] Compound **10** was synthesized by treatment of **7i** with HCl/dioxane to remove the formyl and Boc groups simultaneously. Compound **11a** was then obtained after saponification. In an alternative procedure, compound **11b** was synthesized by the treatment of **9l** with HCl/dioxane. The binding affinities of compounds **11** have shown that the formyl group is not necessary to form the interaction with Mdm2.
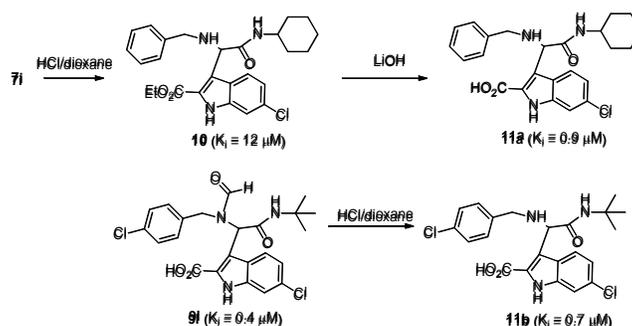
Scheme 4. Synthesis of compounds **11**

We have previously demonstrated that the introduction of solubilizing substituents can improve the water solubility of imidazolines and imidazoles while retaining or improving p53-Mdm2 inhibitory activity.[92, 123, 125] Thus compound **13** was synthesized by amine coupling with compound **8e** in the presence of 1,5,7-triazabicyclo[4,4,0]dec-5-ene (TBD, **Scheme 5**). The transformation into amide, however, leads to a decrease in the binding affinity by 2-3 fold.
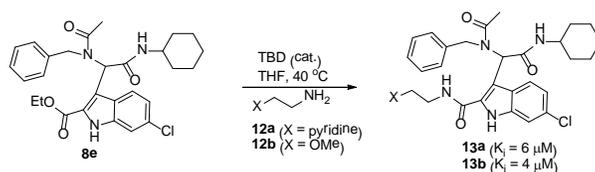
Scheme 5. Synthesis of compounds **13**

The enantiomers of compound **8l** were efficiently separated using preparative supercritical fluid chromatography (SFC), and transformed to the corresponding enantiomers of compound **9l** (**Appendix B**). The enantiomer (+)-**9l** (K_i = 300 nM), is more potent than enantiomer (-)-**9l** (K_i = 700 nM). NMR experiments using [^{1}H,^{15}N] HSQC were performed with Mdm2 titrated against the enantiomer (+)-**9l** (**Figure 12**). Strong binding of (+)-**9l** to Mdm2 was indicated by the signals doubling.[133, 134] The NMR spectra of **9l**-Mdm2 titration also showed a slow chemical exchange.
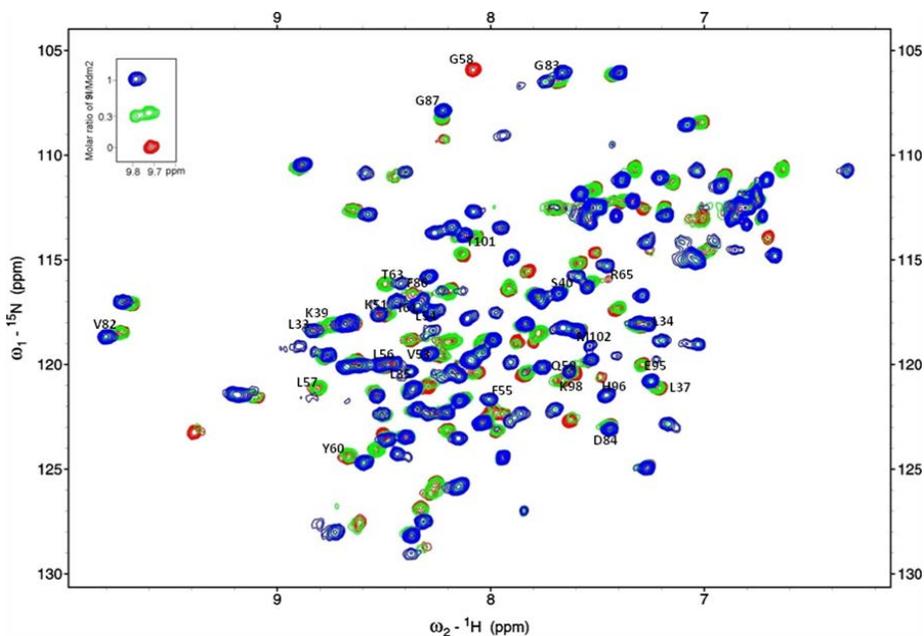
Figure 12. Superposition of NMR HSQC spectra of ^{15}N-labeled Mdm2 titrated against the stronger binding enantiomer (+)-**9l**. The spectrum of free Mdm2 is shown in red. The spectrum of (+)-**9l**-Mdm2 (intermediate ratio, 3:10) is shown in green, and the spectrum of (+)-**9l**-Mdm2 (final ratio, 1:1) is shown in blue. The inset shows the cross-peaks at ca. 118.5 ppm in the ^{15}N dimension and 9.7 ppm in the ^{1}H dimension, which demonstrates tight binding of (+)-**9l** to Mdm2.

We were able to further investigate the binding mechanism of the most potent compound **9l** and Mdm2 using crystallography. The enantiomer (*S*)-**9l** co-crystallized and binds into the well established p53-Mdm2 binding pocket (**Figure 13**).[90] The indole anchor of (*S*)-**9l** binds in the Trp23 pocket of Mdm2. The good alignment of the two indoles of (*S*)-**9l** and Trp23 (RMSD = 0.65 Å) is consistent with the prediction of the ANCHOR.QUERY approach. As shown in the p53-Mdm2 structure, the indole fragment of (*S*)-**9l** forms a hydrogen bond to the carbonyl of Leu54 (2.95 Å). The hydrophobic amino acids make various van der Waals contacts, e.g. the Leu54 methyl group forms short C-H…π interactions with the indole fragment. The *tert*-butyl amide fragment of (*S*)-**9l** resides in the Phe19 pocket and has many hydrophobic contacts with

the surrounding amino acids including a short contact to the sulfur of Met62 (2.85 Å). The 4-chlorobenzyl fragment deeply inserts into the Leu26 pocket forming several hydrophobic contacts. Noteworthy is the short distance between the phenyl group to the adjacent His96, pointing to π-stacking interactions (3.1-3.5 Å). Similar π-stacking contacts with Mdm2 are observed in the spirocyclic indolone, the benzodiazepinedione and the chromeno-triazolopyrimidine structures, but not in the imidazole and nutlin structures.[27, 28, 86-88] The two amide groups of (*S*)-**9l** do not have hydrogen bond contacts to the receptor. The formyl group rather points towards the solvent space, indicating that the introduction of solubilizing or affinity-enhancing groups at this position might be useful for further optimization.
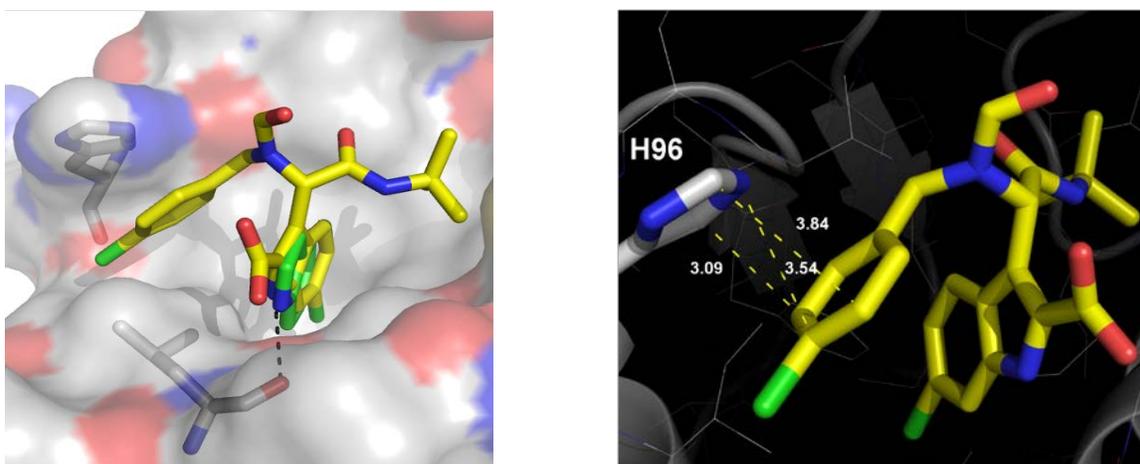
Figure 13. The cocrystal structure of (*S*)-**9l** (yellow sticks) in Mdm2 (grey, blue and red surface with the amino acids His96 and Leu54 shown as sticks). For comparison, the indole ring of Trp23 from the p53-Mdm2 complex (PDB ID: 1YCR) is aligned and shown in green sticks. The hydrogen bond between the indole fragment and the carbonyl of Leu54 is indicated by a black dotted line (d = 2.95 Å). The structural data is available from the PDB (ID: 3TJ2, resolution: 1.9 Å).

The lead compound (+)-**9l** (molecular weight: 475 Da) has a binding efficiency index (BEI) of 13.7, which indicates the best ligand efficiency compared with other published and cocrystallized p53-Mdm2 inhibitors. The water solubility of **9l** is measured as 1.3 mg/mL. These properties suggest reasonable drug-like properties of a lead compound that can be further optimized.

In summary, a novel acyclic scaffold useful for antagonizing p53-Mdm2 interaction, a key anticancer target is described. The scaffold was discovered by a novel web-based pharmacophore search approach using ANCHOR.QUERY. The scaffold is based on an efficiently accessible Ugi-4CR backbone, which leads to a rapid SAR elucidation. The initial hit compounds with micromolar affinity were optimized to a potent lead compound (+)-**9l** with 300 nM affinity. The structural basis of the interaction was elucidated using co-crystal structure analysis: the compound binds in the well-known hotspot pocket in Mdm2 occupied by the p53 triad (Phe19, Trp23, Leu26).

Materials and Methods

3-Formylindoles **1-3** were prepared from the corresponding indoles using standard conditions (Vilsmeier-Haack formylation, *N*-Boc-protection). The purification was conducted using preparative silica gel TLC plates (1000 μm, 20cm×20cm).

Method A: The mixture of aldehyde (**1** or **2**, 0.2 mmol), isocyanide (**4**, 0.2 mmol), amine (**5**, 0.2 mmol), acid (**6**, 0.2 mmol) in 0.5 mL of methanol was stirred at RT for 2 days. The Ugi product (**7**) was purified by chromatography on silica gel (petroleum ether/ethyl acetate, 3:1). The Ugi product (**7**) was treated with 0.5 mL of DCM and 100 μL of TFA, and stirred overnight at RT. After evaporation of the solvent, the product (**8**) was purified by chromatography on silica gel (petroleum ether/ethyl acetate, 1:2).

tert-**Butyl 3-(2-(benzylamino)-1-(*N*-isobutylacetamido)-2-oxoethyl)-1*H*-indole-1-carboxylate (7a):** off-white solid (44 mg, yield: 46%). HPLC/MS: t_R = 12.04 min; m/z = 478.1 [M+H]$^+$. HRMS: $C_{28}H_{35}N_3O_4$, [M+Na]$^+$; 500.2525 (calcd.), 500.2558 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.72 (d, 1H, J = 6.6 Hz), 0.76 (d, 1H, J = 6.6 Hz), 1.68 (s, 9H), 1.86 (m, 1H), 2.22 (s,

3H), 3.02-3.13 (m, 2H), 4.41-4.54 (m, 2H), 6.20 (s, 1H), 7.13 (s, 1H), 7.22-7.37 (m, 8H), 8.14 (s, 1H), 8.20 (m, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 19.7, 20.1, 22.4, 27.8, 28.2, 43.5, 54.5, 54.9, 84.1, 113.5, 115.5, 118.4, 123.0, 124.9, 127.2, 127.3, 127.7, 128.6, 129.7, 135.1, 138.1, 149.5, 169.8, 172.5.

N-Benzyl-2-(1H-indol-3-yl)-2-(N-isobutylacetamido)acetamide (8a): off-white solid (19 mg, yield: 55%). HPLC/MS: t_R = 10.30 min; m/z = 378.2 [M+H]$^+$. HRMS: C$_{23}$H$_{27}$N$_3$O$_2$, [M+Na]$^+$; 400.2001 (calcd.), 400.1994 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.71 (d, 1H, J = 6.6 Hz), 0.77 (d, 1H, J = 6.6 Hz), 1.84 (m, 1H), 2.21 (s, 3H), 3.07-3.09 (m, 2H), 4.35-4.54 (m, 2H), 6.06 (s, 1H), 6.91 (s, 1H), 7.14-7.28 (m, H), 7.41 (d, 1H, J = 7.8 Hz), 7.48 (d, 1H, J = 7.8 Hz), 7.74 (s, 1H), 8.79 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 19.7, 20.1, 22.4, 27.8, 43.5, 55.5, 55.6, 109.8, 111.6, 118.1, 120.2, 122.5, 126.1, 127.2, 127.6, 128.6, 135.6, 138.2, 170.6, 172.1.

N-Benzyl-2-(N-benzylacetamido)-2-(1H-indol-3-yl)acetamide (8b): off-white solid (26 mg, yield: 46%). HPLC/MS: t_R = 10.35 min; m/z = 412.2 [M+H]$^+$. HRMS: C$_{26}$H$_{25}$N$_3$O$_2$, [M+Na]$^+$; 434.1844 (calcd.), 434.1812 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 2.05 (s, 3H), 4.38-4.53 (m, 2H), 4.63-4.67 (m, 2H), 6.53 (s, 1H), 6.60 (m, 1H), 6.90 (m, 2H), 7.04-7.06 (m, 3H), 7.13 (m, 1H), 7.21 (m, 1H), 7.24-7.32 (m, 5H), 7.52 (m, 1H), 7.57 (m, 1H), 8.49 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 22.6, 43.6, 50.1, 54.4, 109.2, 111.5, 118.6, 120.3, 122.6, 126.0, 126.1, 126.7, 127.1, 127.4, 127.7, 128.3, 128.6, 135.8, 137.7, 138.0, 170.2, 172.8

1-tert-Butyl 2-ethyl 3-(2-(benzylamino)-1-(N-isobutylacetamido)-2-oxoethyl)-6-chloro-1H-indole-1,2-dicarboxylate (7c): yellow solid (32 mg, yield: 27%). HPLC/MS: t_R =12.38 min; m/z = 583.9 [M+H]$^+$. HRMS: C$_{31}$H$_{38}$N$_3$O$_6$Cl, [M+Na]$^+$; 606.2347 (calcd.), 606.2401 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.58 (d, 3H, J = 6.0 Hz), 0.75 (d, 3H, J = 6.6 Hz), 1.37 (t, 3H, J = 7.2 Hz), 1.46 (m, 1H), 1.65 (s, 9H), 2.22 (s, 3H), 3.24 (d, 2H, J = 7.2 Hz), 4.35-4.49 (m, 4H), 6.08

(s, 1H), 6.30 (s, 1H), 7.18-7.26 (m, 6H), 7.74 (m, 1H), 8.14 (m, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 13.9, 14.0, 19.90, 19.91, 22.1, 27.9, 28.5, 43.9, 54.6, 54.7, 62.5, 86.1, 115.3, 117.3, 122.7, 124.5, 125.7, 127.4, 127.9, 128.6, 131.4, 132.8, 136.2, 137.7, 148.4, 161.9, 168.5, 172.2.

Ethyl 3-(2-(benzylamino)-1-(*N*-isobutylacetamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (8c): yellowish solid (17 mg, yield: 64%). HPLC/MS: t_R = 11.01 min; *m/z* = 484.0 [M+H]$^+$. HRMS: C$_{26}$H$_{30}$ClN$_3$O$_4$, [M+Na]$^+$; 506.1823 (calcd.), 506.1801 (found). ^{1}H NMR (600 MHz, CD$_3$OD, a mixture of rotamers): 0.17 (d, 2H, *J* = 6.6 Hz), 0.39 (d, 3H, *J* = 6.0 Hz), 0.52 (d, 2H, *J* = 6.6 Hz), 0.65 (d, 3H, *J* = 6.6 Hz), 1.39 (t, 3H, *J* = 7.2 Hz), 1.43 (t, 2H, *J* = 7.2 Hz), 2.24 (s, 3H), 2.29 (s, 2H), 2.84 (m, 1H), 3.24-3.33 (m, 4H), 3.69 (m, 1H), 4.36-4.42 (m, 7H), 6.65 (s, 1H), 6.94-6.98 (m, 3H), 7.14-7.23 (m, 8H), 7.30-7.41 (m, 3H), 7.51 (m, 2H), 7.58 (m, 1H). ^{13}C NMR (150 MHz, CD$_3$OD, a mixture of rotamers): 13.3, 13.4, 18.6, 18.8, 20.9, 27.7, 28.4, 41.3, 42.9, 43.2, 51.7, 54.2, 55.0, 57.3, 61.0, 111.9, 112.0, 114.2, 115.0, 121.3, 121.4, 121.6, 122.1, 124.7, 126.3, 126.6, 126.9, 127.0, 127.2, 127.7, 127.9, 128.0, 128.2, 130.7, 130.8, 136.4, 136.6, 138.2, 138.3, 160.9, 161.3, 162.2, 171.0, 171.4, 173.0, 173.3.

1-*tert*-Butyl 2-ethyl 3-(1-(*N*-benzylacetamido)-2-(benzylamino)-2-oxoethyl)-6-chloro-1*H*-indole-1,2-dicarboxylate (7d): yellow solid (49 mg, yield: 40%). HPLC/MS: t_R = 12.13 min; *m/z* = 618.0 [M+H]$^+$. HRMS: C$_{34}$H$_{36}$N$_3$O$_6$Cl, [M+Na]$^+$; 640.2190 (calcd.), 640.2181 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.37 (t, 3H, *J* = 7.2 Hz), 1.59 (s, 9H), 2.12 (s, 3H), 4.35-4.54 (m, 4H), 4.76-4.84 (m, 2H), 6.20 (s, 1H), 6.67 (s, 1H), 6.81 (m, 2H), 6.98-7.00 (m, 3H), 7.17-7.37 (m, 6H), 7.64 (m, 1H), 7.94 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 13.9, 22.1, 27.8, 44.0, 50.4, 53.4, 62.4, 85.7, 115.1, 116.9, 121.9, 124.4, 125.5, 126.0, 126.6, 127.5, 127.88, 127.92, 128.6, 131.1, 132.7, 136.0, 137.0, 137.5, 137.5, 148.1, 161.7, 168.4, 172.5.

Ethyl 3-(1-(*N*-benzylacetamido)-2-(benzylamino)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (8d): off-white solid (18 mg, yield: 44%). HPLC/MS: t_R = 10.97 min; m/z = 518.0 [M+H]$^+$. HRMS: $C_{29}H_{28}N_3O_4Cl$, [M+Na]$^+$; 540.1666 (calcd.), 540.1695 (found). ^{1}H NMR (600 MHz, CD$_3$OD, a mixture of rotamers): 1.37 (t, 2H, J = 7.2 Hz), 1.41 (t, 3H, J = 7.2 Hz), 2.23 (s, 3H), 2.39 (s, 2H), 4.33-4.45 (m, 7H), 4.85 (m, 1H), 6.28 (m, 1H), 6.60 (m, 1H), 6.66 (s, 1H), 6.81 (m, 1H), 6.89 (m, 1H), 6.95-6.99 (m, 4H), 7.12 (s, 1H), 7.19-7.25 (m, 5H), 7.31 (m, 1H), 7.44 (m, 1H), 7.57 (m, 1H). ^{13}C NMR (150 MHz, CD$_3$OD, a mixture of rotamers): 13.3, 13.4, 20.8, 42.9, 43.2, 50.2, 54.4, 60.6, 60.8, 111.7, 111.8, 113.1, 113.5, 121.2, 121.3, 121.6, 124.5, 125.2, 125.5, 125.6, 126.2, 126.6, 126.8, 127.0, 127.3, 127.4, 127.8, 127.9, 128.0, 128.1, 130.38, 130.40, 136.4, 137.5, 138.1, 138.3, 160.5, 160.8.

1-*tert*-Butyl 2-ethyl 3-(1-(*N*-benzylacetamido)-2-(cyclohexylamino)-2-oxoethyl)-6-chloro-1*H*-indole-1,2-dicarboxylate (18e): yellowish solid (44 mg, yield: 36%). HPLC/MS: t_R = 12.91 min; m/z = 610.1 [M+H]$^+$. HRMS: $C_{33}H_{40}N_3O_6Cl$, [M+Na]$^+$; 632.2503 (calcd.), 632.2499 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.94-1.35 (m, 6H), 1.39 (t, 3H, J = 7.2 Hz), 1.60 (s, 9H), 1.66-2.09 (m, 4H), 2.17 (s, 3H), 3.78 (m, 1H), 4.39 (m, 2H), 4.72-4.82 (m, 2H), 5.54 (m, 1H), 6.55 (s, 1H), 6.83 (m, 2H), 7.01 (m, 3H), 7.22-7.28 (m, 1H), 7.72 (m, 1H), 7.96 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 13.9, 22.1, 24.8, 24.9, 25.4, 27.8, 32.7, 32.9, 49.0, 50.5, 53.3, 62.4, 85.7, 115.1, 117.1, 122.0, 124.3, 125.5, 126.5, 127.9, 131.2, 132.6, 136.1, 137.1, 148.2, 161.7, 167.3, 172.2.

Ethyl 3-(1-(*N*-benzylacetamido)-2-(cyclohexylamino)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (8e): off-white solid (16 mg, yield: 44%). HPLC/MS: t_R = 11.26 min; m/z = 509.9 [M+H]$^+$. HRMS: $C_{28}H_{32}N_3O_4Cl$, [M+Na]$^+$; 532.1979 (calcd.), 532.1959 (found). ^{1}H NMR (600 MHz, CD$_3$OD, a mixture of rotamers): 0.98-1.40 (m, 6H), 1.40 (m, 3H), 1.44 (m, 3H), 1.63-1.91

(m, 10H), 2.21 (s, 3H), 2.38 (s, 2H), 3.75-3.85 (m, 3H), 4.34-4.42 (m, 4H), 4.83 (m, 2H), 5.31 (m, 1H), 6.32 (m, 1H), 6.57-7.15 (m, 10H), 7.34 (m, 1H), 7.75 (m, 2H), 7.89 (m, 1H), 8.12 (m, 1H). ^{13}C NMR (150 MHz, CD$_3$OD, a mixture of rotamers): 12.12, 12.17, 19.4, 19.6, 23.49, 23.54, 23.6, 23.7, 23.86, 23.92, 30.9, 30.98, 31.01, 31.2, 47.6, 47.7, 49.0, 53.0, 55.6, 59.4, 59.5, 110.6, 110.7, 112.1, 112.4, 119.9, 129.97, 120.02, 120.3, 123.3, 123.4, 124.1, 124.2, 124.4, 124.9, 125.6, 126.2, 126.4, 126.9, 129.2, 129.3, 135.1, 136.4, 137.3, 159.2, 159.6.

Ethyl 6-chloro-3-(1-(*N*-(4-chlorobenzyl)acetamido)-2-(cyclohexylamino)-2-oxoethyl)-1*H*-indole-2-carboxylate (8f): off-white solid (32 mg, yield: 71%). HPLC/MS: t_R = 11.64 min; *m/z* = 543.9 [M+H]$^+$. HRMS: C$_{28}$H$_{31}$Cl$_2$N$_3$O$_4$, 543.16916 (calcd.), 543.168429 (found). ^{1}H NMR (600 MHz, CD$_3$OD, a mixture of rotamers): 0.93-1.36 (m, 6H), 1.38 (t, 3H, *J* = 7.2 Hz), 1.42 (t, 3H, *J* = 7.2 Hz), 1.59-1.89 (m, 8H), 2.22 (s, 2H), 2.37 (s, 3H), 3.79 (m, 2H), 4.34-4.40 (m, 4H), 4.81 (m, 1H), 5.25 (m, 1H), 6.28 (m, 2H), 6.55 (m, 2H), 6.82 (d, 2H, *J* = 7.8 Hz), 6.94 (d, 2H, *J* = 7.8 Hz), 7.12 (m, 1H), 7.16 (m, 1H), 7.35 (s, 1H), 7.40 (s, 1H), 7.74 (m, 2H), 7.91 (d, 1H, *J* = 7.8 Hz), 8.16 (d, 1H, *J* = 7.2 Hz). ^{13}C NMR (150 MHz, CD$_3$OD, a mixture of rotamers): 13.35, 13.39, 20.6, 20.8, 24.7, 24.75, 24.79, 24.9, 25.08, 25.13, 32.1, 32.2, 32.3, 32.4, 32.5, 49.0, 49.1, 49.7, 54.2, 56.8, 60.7, 60.8, 111.9, 112.1, 113.4, 113.7, 121.2, 121.3, 121.4, 121.5, 124.5, 125.3, 126.2, 126.8, 127.0, 127.4, 127.5, 128.0, 130.5, 130.7, 131.3, 131.8, 136.3, 136.4, 136.5, 137.5, 160.4, 160.7.

Ethyl 6-chloro-3-(2-(cyclohexylamino)-1-(*N*-(3,4-dichlorobenzyl)acetamido)-2-oxoethyl)-1*H*-indole-2-carboxylate (8g): off-white solid (10 mg, yield: 35%). HPLC/MS: t_R = 11.93 min; *m/z* = 577.9 [M+H]$^+$. HRMS: C$_{28}$H$_{30}$Cl$_3$N$_3$O$_4$, 577.13019 (calcd.), 577.129139 (found). ^{1}H NMR (600 MHz, *d*^{6}-DMSO, a mixture of rotamers): 0.85-1.25 (m, 14H), 1.33-1.38 (m, 5H), 1.50-1.75 (m, 14H), 2.00 (s, 2H), 2.20 (s, 2H), 3.16 (s, 3H), 3.57-3.74 (m, 6H), 4.27-4.36 (m, 4H), 4.77 (m,

1H), 5.07 (m, 1H), 6.29 (m, 2H), 6.44 (m, 1H), 6.63-6.84 (m, 2H), 7.11-7.39 (m, 6H), 7.68-8.13 (m, 5H). ^{13}C NMR (150 MHz, d^6-DMSO, a mixture of rotamers): 14.6, 14.8, 22.0, 22.3, 24.8, 24.9, 25.0, 25.5, 25.6, 32.65, 32.76, 32.81, 47.0, 48.4, 48.5, 49.1, 49.4, 53.9, 56.5, 61.30, 61.33, 112.5, 112.7, 114.5, 114.7, 121.5, 121.9, 122.1, 122.4, 124.9, 125.8, 126.2, 127.3, 127.7, 128.5, 129.0, 129.6, 129.8, 130.1, 130.2, 130.8, 136.6, 140.5, 140.9, 160.4, 169.0, 171.2.

Method B: The mixture of aldehyde (**2**, 0.2 mmol), isocyanide (**4**, 0.2 mmol), amine (**5**, 0.2 mmol), acid (**6**, 0.2 mmol) in 0.5 mL of methanol was stirring under RT for 2 days. The products (**7** and **8**) were purified by chromatography on silica gel (petroleum ether/ethyl acetate, 3:1).

1-*tert*-Butyl 2-ethyl 3-(1-(*N*-benzylbutyramido)-2-(cyclohexylamino)-2-oxoethyl)-6-chloro-1*H*-indole-1,2-dicarboxylate (7h): yellow solid (58 mg, yield: 46%). HPLC/MS: t_R = 13.53 min; m/z = 638.0 [M+H]$^+$. HRMS: $C_{35}H_{44}ClN_3O_6$, 637.29186 (calcd.), 637.290037 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.90-1.38 (m, 14H), 1.59 (s, 9H), 1.67-2.46 (m, 8H), 3.78 (m, 1H), 4.37 (m, 2H), 4.71-4.84 (ABd, 2H, J = 18.0 Hz), 5.63 (m, 1H), 6.60 (s, 1H), 6.79 (m, 2H), 6.98 (m, 3H), 7.20-7.22 (m, 1H), 7.71 (m, 1H), 7.93 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 13.9, 14.0, 18.9, 24.78, 24.84, 25.4, 27.8, 32.7, 32.9, 35.5, 49.0, 49.6, 53.2, 62.3, 85.5, 115.0, 117.3, 122.0, 124.2, 125.5, 126.1, 126.4, 127.8, 131.1, 132.6, 136.1, 137.3, 148.2, 161.7, 167.5, 174.6.

Ethyl 3-(1-(*N*-benzylbutyramido)-2-(cyclohexylamino)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (8h): yellow solid (37 mg, yield: 34%). HPLC/MS: t_R = 11.93 min; m/z = 538.1 [M+H]$^+$. HRMS: $C_{30}H_{36}ClN_3O_4$, 537.23943 (calcd.), 537.239259 (found). ^{1}H NMR (600 MHz, CDCl$_3$, major rotamer): 0.92-1.06 (m, 8H), 1.28-1.39 (m, 6H), 1.57-1.93 (m, 10H), 2.27-2.45 (m, 2H), 3.85 (m, 1H), 4.30-4.31 (m, 2H), 4.47 (ABd, 1H, J = 18.0 Hz), 4.78 (ABd, 1H, J = 18.0 Hz), 5.56 (m, 1H), 6.53 (m, 1H), 6.64 (m, 2H), 6.97-7.24 (m, 7H), 7.84 (m, 1H), 9.32 (m, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, major rotamer): 13.9, 14.3, 18.9, 24.8, 24.9, 25.4, 32.90, 32.94,

35.6, 48.8, 49.6, 54.2, 61.6, 111.9, 114.5, 122.3, 122.8, 125.0, 125.7, 126.5, 126.6, 127.3, 127.7, 127.8, 131.5, 135.9, 138.1, 160.9, 169.0, 174.2.

1-*tert*-Butyl 2-ethyl 3-(1-(*N*-benzylformamido)-2-(cyclohexylamino)-2-oxoethyl)-6-chloro-1*H*-indole-1,2-dicarboxylate (7i): yellow solid (75 mg, yield: 63%). HPLC/MS: t_R = 12.89 min; m/z = 595.9 [M+H]$^+$. HRMS: $C_{32}H_{38}ClN_3O_6Na$, 618.2347 (calcd.), 618.2329 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 0.85-1.06 (m, 6H), 1.27-1.39 (m, 7H), 1.59-1.65 (m, 20H), 1.76-1.92 (m, 3H), 3.75 (m, 2H), 4.24-4.66 (m, 6H), 5.40 (s, 1H), 5.72- 5.91 (m, 2H), 6.33 (s, 1H), 6.77-7.05 (m, 6H), 7.16-7.20 (m, 4H), 7.48-7.62 (m, 2H), 7.93 (s, 1H), 8.01 (s, 1H), 8.35 (s, 1H), 8.44 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 13.9, 14.0, 24.65, 24.73, 24.80, 24.84, 25.3, 25.4, 27.8, 27.9, 32.4, 32.6, 32.7, 32.9, 46.3, 49.0, 49.1, 49.8, 51.0, 56.4, 62.3, 85.7, 86.0, 115.0, 115.3, 116.4, 117.7, 121.8, 122.1, 124.2, 124.6, 125.1, 125.9, 126.6, 127.1, 127.4, 127.8, 128.0, 128.4, 130.4, 131.0, 132.7, 133.1, 136.0, 136.3, 136.4, 136.5, 148.1, 148.3, 161.6, 161.7, 163.7, 163.8, 166.2, 166.4.

Ethyl 3-(1-(*N*-benzylformamido)-2-(cyclohexylamino)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (8i): yellowish solid (27 mg, yield: 27%). HPLC/MS: t_R = 16.99 min; m/z = 496.3 [M+H]$^+$. HRMS: $C_{27}H_{30}ClN_3O_4Na$, 518.1823 (calcd.), 518.1794 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 0.89-1.31 (m, 12H), 1.33-1.38 (m, 6H), 1.59-1.94 (m, 12H), 3.82 (m, 2H), 4.30-4.72 (m, 8H), 5.59-5.72 (m, 2H), 6.20 (s, 1H), 6.59 (m, 2H), 6.77 (s, 1H), 6.97-7.26 (m, 14H), 7.52 (m, 1H), 7.79 (m, 1H), 8.44 (s, 1H), 8.52 (s, 1H), 9.26 (s, 1H), 9.58 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.3, 14.4, 24.75, 24.79, 24.9, 25.3, 25.4, 32.6, 32.7, 32.8, 32.9, 33.0, 46.4, 48.9, 50.1, 52.2, 57.3, 61.6, 61.7, 111.9, 112.3, 113.5, 115.2, 122.3, 122.4, 122.5, 122.7, 124.9, 125.6, 125.9, 126.3, 127.1, 127.26, 127.28, 127.69, 127.72,

127.79, 127.84, 128.3, 128.8, 131.7, 131.8, 135.9, 136.0, 137.3, 137.4, 160.4, 160.6, 160.7, 161.1, 163.7, 164.7, 167.9, 168.1.

Method C: The mixture of aldehyde (**3**, 0.2 mmol), isocyanide (**4**, 0.2 mmol), amine (**5**, 0.2 mmol), acid (**6**, 0.2 mmol) in 0.5 mL of methanol was stirred at RT for 5-7 days. The product (**8**) was purified by chromatography on silica gel (petroleum ether/ethyl acetate, 1:1).

Ethyl 6-chloro-3-(1-(*N*-(4-chlorobenzyl)formamido)-2-(cyclohexylamino)-2-oxoethyl)-1*H*-indole-2-carboxylate (8j): yellow solid (55 mg, yield: 52%). HPLC/MS: t_R = 17.00 min; m/z = 530.1 [M+H]$^+$. HRMS: $C_{27}H_{29}N_3O_4Cl_2Na$, 552.1433 (calcd.), 552.1401 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 0.86-1.07 (m, 6H), 1.31-1.39 (m, 9H), 1.58-1.87 (m, 10H), 3.65-3.84 (m, 3H), 4.25 (ABd, 1H, J = 15.0 Hz), 4.30-4.35 (m, 4H), 4.59 (ABd, 1H, J = 16.8 Hz), 4.85 (ABd, 1H, J = 15.6 Hz), 5.57 (m, 1H), 5.65 (m, 1H), 6.18 (s, 1H), 6.51 (m, 1H), 6.76 (s, 1H), 6.79-7.28 (m, 9H), 7.56 (m, 1H), 7.79 (m, 1H), 8.41 (s, 1H), 8.48 (s, 1H), 9.34 (s, 1H), 9.58 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.3, 24.7, 24.8, 25.3, 25.4, 32.7, 32.8, 45.9, 49.0, 49.5, 52.1, 56.9, 61.7, 112.0, 112.4, 113.4, 114.9, 122.1, 122.5, 122.6, 124.7, 125.5, 126.4, 127.17, 127.23, 127.3, 127.8, 128.1, 128.2, 128.8, 129.1, 131.8, 131.9, 132.8, 135.86, 135.89, 136.0, 160.5, 160.6, 163.6, 164.6, 167.8, 168.0.

Ethyl 6-chloro-3-(2-(cyclohexylamino)-1-(*N*-(4-fluorobenzyl)formamido)-2-oxoethyl)-1*H*-indole-2-carboxylate (8k): yellow solid (56 mg, yield: 55%). HPLC/MS: t_R = 16.73 min; m/z = 514.3 [M+H]$^+$. HRMS: $C_{27}H_{29}N_3O_4ClFNa$, 536.1728 (calcd.), 536.1700 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 0.88-1.19 (m, 8H), 1.32-1.41 (m, 10H), 1.59-1.96 (m, 14H), 3.85 (m, 3H), 4.27 (ABd, 1H, J = 15.0 Hz), 4.34-4.38 (m, 4H), 4.60 (ABd, 1H, J = 16.2 Hz), 4.88 (ABd, 1H, J = 15.6 Hz), 5.48 (m, 1H), 5.58 (m, 1H), 6.17 (s, 1H), 6.55-6.86 (m, 8H), 7.17 (m, 2H), 7.32 (m, 1H), 7.59 (m, 1H), 7.83 (m, 1H), 8.42 (s, 1H), 8.50 (s, 1H), 9.06 (s, 1H),

9.24 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.38, 14.40, 24.7, 24.8, 24.9, 25.3, 25.39, 25.41, 32.7, 32.8, 32.9, 33.0, 33.1, 45.7, 49.0, 49.5, 52.0, 56.8, 61.7, 61.8, 111.8, 112.1, 113.6, 114.5, 114.6, 114.7, 114.9, 115.3, 122.3, 122.6, 122.7, 122.8, 124.8, 125.6, 126.3, 127.1, 127.4, 127.5, 129.09, 129.14, 131.9, 132.1, 133.2, 135.7, 135.8, 160.3, 160.5, 163.5, 164.5, 167.7, 167.8.

Ethyl 3-(2-(*tert*-butylamino)-1-(*N*-(4-chlorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (8l): yellow solid (70 mg, yield: 70%). HPLC/MS: t_R = 16.83 min; m/z = 504.4 [M+H]$^+$. HRMS: C$_{25}$H$_{27}$N$_3$O$_4$Cl$_2$Na, 526.1276 (calcd.), 526.1237 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.25 (s, 9H), 1.30 (s, 7H), 1.35-1.39 (m, 10 H), 4.22 (ABd, 1H, J = 15.0 Hz), 4.29-4.45 (m, 4H), 4.58 (ABd, 1H, J = 16.2 Hz), 4.94 (ABd, 1H, J = 15.6 Hz), 5.54 (s, 1H), 6.11 (s, 1H), 6.45 (m, 1H), 6.71 (s, 1H), 6.82-7.28 (m, 9H), 7.32 (s, 1H), 7.65 (m, 1H), 7.87 (m, 1H), 8.41 (s, 1H), 8.47 (s, 1H), 9.53 (s, 1H), 9.79 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.36, 14.39, 28.4, 28.6, 28.9, 30.8, 46.1, 49.5, 51.9, 52.1, 52.5, 57.1, 61.7, 112.0, 112.3, 115.2, 122.3, 122.5, 122.6, 122.7, 124.7, 126.3, 127.1, 127.2, 127.7, 128.1, 128.6, 128.8, 128.9, 129.1, 129.7, 131.7, 131.9, 132.7, 132.8, 135.9, 136.0, 160.56, 160.64, 163.5, 164.6, 168.0, 168.1.

Ethyl 3-(2-(*tert*-butylamino)-1-(*N*-(3,4-dichlorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (8m): yellow solid (77 mg, yield: 72%). HPLC/MS: t_R = 11.93 min; m/z = 538.2 [M+H]$^+$. HRMS: C$_{25}$H$_{26}$Cl$_3$N$_3$O$_4$Na, 560.0887 (calcd.), 560.0878 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.30 (s, 9H), 1.31 (s, 6H), 1.37-1.41 (m, 6H), 4.13 (ABd, 1H, J = 15.6 Hz), 4.30-4.36 (m, 4H), 4.59 (m, 1H), 5.03 (ABd, 1H, J = 15.6 Hz), 5.60 (m, 1H), 6.16 (s, 1H), 6.30-6.76 (m, 4H), 6.94-7.19 (m, 4H), 7.35 (m, 1H), 7.68 (m, 1H), 7.86 (m, 1H), 8.42 (s, 1H), 8.46 (s, 1H), 9.66 (m, 1H), 9.89 (m, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a

mixture of rotamers): 14.4, 28.5, 28.6, 45.5, 49.2, 52.0, 52.2, 56.9, 61.8, 61.9, 112.2, 112.4, 113.1, 114.8, 122.0, 122.4, 113.1, 114.8, 122.0, 122.4, 122.7, 122.8, 124.6, 124.9, 125.4, 126.4, 126.6, 127.2, 127.5, 128.9, 129.4, 129.6, 130.5, 130.6, 131.6, 131.9, 132.0, 136.0, 137.7, 138.0, 160.5, 160.6, 163.5, 164.6, 168.1.

Ethyl 3-(2-(*tert*-butylamino)-1-(*N*-(2,4-dichlorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (8n): yellow solid (92 mg, yield: 86%). HPLC/MS: t_R = 11.58 min; m/z = 537.8 [M+H]$^+$. HRMS: $C_{25}H_{26}N_3O_4Cl_3Na$, 560.0887 (calcd.), 560.0880 (found). ^{1}H NMR (400 MHz, CDCl$_3$, a mixture of rotamers): 1.26 (s, 9H), 1.29 (s, 5H), 1.35-1.39 (m, 6H), 4.30-4.39 (m, 3H), 4.48-4.61 (m, 3H), 4.96 (ABd, 1H, J = 16.4 Hz), 5.59 (s, 1H), 6.18 (s, 1H), 6.73 (s, 1H), 6.82-6.89 (m, 2H), 6.95-7.12 (m, 5H), 7.31 (m, 2H), 7.64 (m, 1H), 7.81 (m, 1H), 8.33 (s, 1H), 8.48 (s, 1H), 9.84 (s, 1H), 10.13 (s, 1H). ^{13}C NMR (100 MHz, CDCl$_3$, a mixture of rotamers): 14.3, 28.5, 28.6, 28.9, 30.9, 39.5, 44.5, 47.5, 51.9, 52.1, 52.9, 57.3, 61.7, 61.8, 112.0, 112.3, 113.4, 115.0, 122.2, 122.5, 122.7, 122.9, 124.4, 125.3, 126.2, 126.4, 126.6, 127.0, 127.4, 128.7, 128.8, 129.1, 129.3, 129.7, 130.7, 131.8, 132.0, 132.8, 133.1, 133.4, 133.8, 134.1, 136.0, 160.8, 161.4, 164.1, 164.5, 167.8.

Ethyl 3-(2-(tert-butylamino)-1-(*N*-((6-chloropyridin-3-yl)methyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (8o): yellow solid (48 mg, 48%). HRMS: $C_{24}H_{27}Cl_2N_4O_4$, 505.1409 (calcd.), 505.1459 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.28 (s, 9H), 1.30 (s, 4H), 1.33-1.36 (m, 6H), 4.22 (ABd, 1H, J = 15.0 Hz), 4.27-4.33 (m, 3H), 4.40 (ABd, 1H, J = 16.8 Hz), 4.62 (ABd, 1H, J = 16.8 Hz), 5.04 (ABd, 1H, J = 15.6 Hz), 6.15 (s, 1H), 6.70 (s, 1H), 6.91 (s, 1H), 7.01 (m, 1H), 7.15 (m, 1H), 7.30 (m, 1H), 7.36 (s, 1H), 7.40 (s, 1H), 7.55 (s, 1H), 7.70 (m, 1H), 7.86 (m, 1H), 8.03 (s, 1H), 8.29 (m, 1H), 8.43 (s, 1H), 8.46 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.3, 28.5, 28.6, 28.9, 30.9, 43.9, 47.1,

50.5, 51.5, 52.0, 52.2, 56.8, 60.5, 61.7, 112.4, 112.6, 113.0, 114.8, 122.0, 122.3, 122.7, 122.8, 123.1, 123.4, 124.4, 125.2, 126.3, 127.1, 132.1, 132.3, 132.4, 136.1, 136.3, 138.5, 147.1, 148.0, 149.7, 160.6, 160.8, 163.1, 163.3, 164.6, 168.1.

Ethyl 3-(2-(tert-butylamino)-1-(*N*-((*S*)-1-(4-chlorophenyl)ethyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (8p): yellow solid (55 mg, yield: 53%). HPLC/MS: t_R = 11.88, 12.12 min; m/z = 518.3 [M+H]$^+$. HRMS: $C_{26}H_{29}Cl_2N_3O_4Na$, 540.1433 (calcd.), 540.1450 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a 5:3 mixture of diastereomers): 1.09 (m, 6H), 1.24-1.47 (m, 28H), 1.74-1.78 (m, 4H), 4.19-4.69 (m, 6H), 5.56-5.67 (m, 2H), 6.46 (m, 2H), 6.70-6.92 (m, 4H), 6.95-7.19 (m, 8H), 7.29-7.37 (m, 6H), 7.45-7.94 (m, 6H), 8.59 (s, 1H), 8.69 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of diastereomers): 14.3, 14.4, 17.3, 17.7, 21.7, 23.4, 28.3, 28.5, 28.6, 28.9, 30.9, 51.4, 51.7, 51.9, 52.0, 53.5, 54.1, 54.6, 55.7, 55.8, 61.6, 61.7, 61.9, 112.1, 112.3, 113.6, 122.6, 122.7, 124.9, 125.1, 125.4, 125.6, 126.1, 127.1, 127.3, 127.9, 128.4, 128.7, 128.8, 129.0, 130.4, 131.8, 131.9, 132.4, 133.6, 133.8, 135.9, 136.2, 138.1, 139.8, 141.6, 160.5, 160.9, 161.1, 162.0, 163.2, 164.8, 167.9, 168.4.

Ethyl 3-(2-(*tert*-butylamino)-1-(*N*-((*R*)-1-(4-chlorophenyl)ethyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (8q): yellow solid (75 mg, yield: 73%). HPLC/MS: t_R = 11.88, 12.12 min; m/z = 518.3 [M+H]$^+$. HRMS: $C_{26}H_{29}Cl_2N_3O_4Na$, 540.1433 (calcd.), 540.1473 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a 5:4 mixture of diastereomers): 1.08 (m, 9H), 1.23-1.48 (m, 30H), 1.74-1.77 (m, 4H), 4.25-4.59 (m, 8H), 5.07 (m, 1H), 5.58-5.68 (m, 3H), 5.99 (s, 1H), 6.46 (m, 2H), 6.70-6.91 (m, 4H), 6.95-7.18 (m, 6H), 7.30-7.38 (m, 6H), 7.45-7.93 (m, 6H), 8.59 (s, 1H), 8.68 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of diastereomers): 14.3, 14.4, 17.3, 17.6, 21.7, 23.4, 28.3, 28.5, 28.6, 51.4, 51.7, 51.9, 52.0, 53.5, 54.1, 54.6, 55.7, 55.8, 61.6, 61.8, 112.2, 112.4, 113.5, 122.4, 122.5, 122.6, 124.8, 125.1, 125.4, 125.6, 126.1, 127.3, 127.6, 127.8,

128.4, 128.6, 128.8, 129.0, 130.4, 131.7, 131.8, 132.4, 133.6, 133.8, 136.0, 136.2, 138.1, 139.8, 141.6, 160.5, 160.9, 161.1, 162.1, 163.2, 164.8, 167.9, 168.4.

Ethyl 3-(2-(*tert*-butylamino)-1-(*N*-(4-hydroxybenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (8r): yellow solid (34 mg, yield: 35%). HPLC/MS: t_R = 10.53 min; m/z = 486.3 [M+H]$^+$. HRMS: $C_{25}H_{28}ClN_3O_5Na$, 508.1615 (calcd.), 508.1633 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.26 (s, 9H), 1.33-1.41 (m, 24 H), 4.11-4.29 (m, 6H), 4.58 (ABd, 1H, *J* = 16.2 Hz), 4.85 (ABd, 1H, *J* = 14.4 Hz), 5.72 (m, 2H), 6.15 (s, 1H), 6.23-7.17 (m, 12H), 7.34-8.27 (m, 6H), 8.44 (s, 1H), 8.47 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.3, 28.4, 28.6, 28.9, 29.7, 30.9, 46.3, 50.0, 50.6, 51.6, 52.1, 52.2, 57.5, 61.6, 61.7, 112.3, 112.4, 112.9, 114.8, 114.9, 115.2, 115.7, 122.3, 122.5, 124.8, 125.5, 126.5, 127.0, 127.5, 128.3, 128.4, 129.0, 129.2, 131.4, 131.8, 136.2, 136.4, 155.7, 155.8, 160.8, 160.9, 163.2, 164.3, 164.9, 168.5, 168.9.

Ethyl 3-(1-(*N*-(biphenyl-4-ylmethyl)formamido)-2-(*tert*-butylamino)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (8s): yellow oil (86 mg, yield: 79%). HPLC/MS: t_R = 12.12 min; m/z = 546.3 [M+H]$^+$. HRMS: $C_{31}H_{32}N_3O_4ClNa$, 568.1979 (calcd.), 568.1971 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.20 (s, 9H), 1.29 (s, 7H), 1.31-1.36 (m, 12H), 4.23-4.59 (m, 8H), 4.87 (ABd, 1H, *J* = 15.0 Hz), 5.65 (m, 2H), 6.16 (s, 1H), 6.59 (m, 2H), 6.73 (s, 1H), 7.08-7.17 (m, 6H), 7.26-7.43 (m, 14H), 7.48-7.56 (m, 4H), 7.62 (m, 1H), 7.85 (m, 1H), 8.43 (m, 1H), 8.52 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.34, 14.37, 28.4, 28.6, 28.9, 30.8, 41.8, 46.5, 49.9, 50.5, 51.9, 52.0, 52.8, 57.6, 61.6, 112.1, 112.4, 112.5, 113.4, 115.4, 122.3, 122.4, 122.8, 124.8, 125.6, 126.3, 126.4, 126.9, 127.0, 127.1, 127.4, 127.5, 128.2, 128.4, 128.8, 131.5, 131.7, 136.1, 136.2, 136.5, 139.9, 140.7, 160.8, 160.9, 161.4, 163.7, 164.8, 168.3.

Ethyl 3-(2-(*tert*-butylamino)-2-oxo-1-(*N*-(4-phenoxybenzyl)formamido)ethyl)-6-chloro-1*H*-indole-2-carboxylate (8t): yellow solid (80 mg, yield: 71%). HPLC/MS: t_R = 12.11 min; m/z = 562.2 [M+H]$^+$. HRMS: $C_{31}H_{32}ClN_3O_5Na$, 584.1928 (calcd.), 584.1909 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.29 (s, 9H), 1.33 (s, 9H), 1.37-1.41 (m, 6H), 4.30-4.38 (m, 6H), 4.61 (ABd, 1H, J = 16.2 Hz), 4.88 (ABd, 1H, J = 15.0 Hz), 5.57 (s, 1H), 5.64 (s, 1H), 6.18 (s, 1H), 6.50 (d, 2H, J = 8.4 Hz), 6.61 (d, 2H, J = 8.4 Hz), 6.74-6.96 (m, 9H), 7.09-7.36 (m, 10H), 7.63 (d, 1H, J = 8.4 Hz), 7.88 (d, 2H, J = 9.0 Hz), 8.47 (s, 1H), 8.52 (s, 1H), 9.39 (s, 1H), 9.69 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.39, 14.44, 28.5, 28.6, 46.1, 49.6, 52.0, 52.1, 52.5, 57.4, 61.7, 61.8, 111.9, 112.3, 113.6, 115.6, 118.4, 118.6, 118.7, 118.9, 119.0, 122.3, 122.4, 122.5, 122.8, 123.2, 123.3, 124.8, 125.6, 126.2, 127.2, 129.2, 129.4, 129.8, 131.7, 131.9, 132.3, 132.4, 135.9, 156.0, 157.1, 157.2, 160.68, 160.72, 163.6, 164.6, 168.19, 168.24.

Method D: The ester **8** was treated with LiOH in EtOH/water (1:1), and stirred at RT for 2 days. The reaction mixture was then acidified with 1M HCl to a pH of ~ 6. The mixture was extracted with DCM (10 mL x 3). The combined organic layer was dried over sodium sulfate, and evaporated.

3-(1-(*N*-Benzylacetamido)-2-(cyclohexylamino)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylic acid (9e): white solid (39 mg, 81%). HPLC/MS: t_R = 11.17 min; m/z = 482.3 [M+H]$^+$. HRMS: $C_{26}H_{28}ClN_3O_4Na$, 504.1666 (calcd.), 504.1681 (found). ^{1}H NMR (600 MHz, CD$_3$OD, a mixture of rotamers): 0.91-1.36 (m, 14H), 1.54-1.86 (m, 10H), 2.04 (s, 3H), 2.40 (s, 3H), 3.71-3.79 (m, 2H), 4.14 (ABd, 1H, J = 15.6 Hz), 4.68 (m, 2H), 5.13 (ABd, 1H, J = 16.2 Hz), 6.54 (m, 2H), 6.95 (m, 4H), 6.99-7.29 (m, 8H), 7.61 (m, 1H), 7.70 (m, 1H). ^{13}C NMR (150 MHz, CD$_3$OD, a mixture of rotamers): 20.8, 21.1, 24.6, 24.7, 24.8, 25.1, 25.2, 32.1, 32.17, 32.23,

32.3, 48.7, 48.9, 50.0, 57.3, 109.2, 111.47, 111.54, 120.07, 120.13, 120.7, 121.3, 125.0, 125.2, 125.3, 125.4, 126.0, 126.8, 127.7, 128.4, 128.6, 135.18, 135.24, 138.1, 138.3, 171.1, 171.3, 173.2.

3-(1-(*N*-Benzylformamido)-2-(cyclohexylamino)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylic acid (9i): yellow solid (18 mg, 91%). HPLC/MS: t_R = 16.91 min; m/z = 468.3 [M+H]$^+$. HRMS: $C_{25}H_{27}N_3O_4Cl$, 468.1690 (calcd.), 468.1690 (found). ^{1}H NMR (600 MHz, CD$_3$OD, a mixture of rotamers): 0.88-1.30 (m, 9H), 1.56-1.84 (m, 9H), 3.63 (m, 2H), 4.26 (m, 2H), 4.68 (ABd, 1H, J = 16.2 Hz), 5.09 (ABd, 1H, J = 15.0 Hz), 6.27 (s, 1H), 6.48 (m, 1H), 6.78-7.08 (m, 9H), 7.32 (s, 1H), 7.34 (s, 1H), 7.64 (m, 1H), 7.66 (m, 1H), 8.36 (s, 1H), 8.42 (s, 1H). ^{13}C NMR (150 MHz, CD$_3$OD, a mixture of rotamers): 24.7, 24.8, 25.08, 25.14, 32.0, 32.2, 46.7, 48.9, 49.9, 52.2, 56.5, 111.7, 111.9, 113.9, 120.9, 121.1, 121.4, 121.5, 124.7, 125.2, 126.4, 126.5, 126.9, 127.2, 127.3, 130.2, 130.4, 136.27, 136.29, 136.8, 137.5, 164.8, 165.2, 169.48, 169.53.

6-Chloro-3-(1-(*N*-(4-chlorobenzyl)acetamido)-2-(cyclohexylamino)-2-oxoethyl)-1*H*-indole-2-carboxylic acid (9j): yellow solid (16 mg, 70%). HPLC/MS: t_R = 11.55 min; m/z = 516.3 [M+H]$^+$. HRMS: $C_{26}H_{27}Cl_2N_3O_4$, 515.137862 (calcd.), 515.137663 (found). ^{1}H NMR (600 MHz, Acetone, a mixture of rotamers): 0.88-1.34 (m, 8H), 1.55-1.95 (m, 8H), 2.29 (s, 3H), 3.32 (s, 2H), 3.73-3.86 (m, 2H), 3.94 (ABd, 1H, J = 15.6 Hz), 4.36 (m, 1H), 4.58 (ABd, 1H, J = 18.0 Hz), 4.80 (ABd, 1H, J = 18.0 Hz), 5.25 (ABd, 1H, J = 15.6 Hz), 6.44 (m, 2H), 6.53 (s, 1H), 6.83 (m, 2H), 6.99-7.55 (m, 6H), 7.84 (m, 1H), 7.94 (m, 1H), 10.92 (s, 1H), 11.02 (s, 1H). ^{13}C NMR (150 MHz, Acetone, a mixture of rotamers): 14.5, 21.1, 21.8, 24.8, 25.3, 32.5, 32.7, 41.9, 47.0, 48.7, 56.6, 64.7, 111.9, 112.2, 120.7, 121.4, 122.2, 123.5, 126.9, 127.4, 127.6, 128.3, 129.2, 130.2, 130.6, 136.3, 138.7, 169.0, 171.0.

6-Chloro-3-(2-(cyclohexylamino)-1-(*N*-(4-fluorobenzyl)formamido)-2-oxoethyl)-1*H*-indole-2-carboxylic acid (9k): yellow solid (45 mg, 92%). HPLC/MS: t_R = 10.96 min; *m/z* = 486.3 [M+H]⁺. HRMS: $C_{25}H_{25}N_3O_4ClFNa$, 508.1415 (calcd.), 508.1419 (found). ¹H NMR (600 MHz, CD₃OD, a mixture of rotamers): 0.92-1.12 (m, 8H), 1.29-1.36 (m, 4H), 1.58-1.90 (m, 8H), 2.13-2.37 (m, 2H), 3.67-3.77 (m, 2H), 4.27-4.32 (m, 2H), 4.69 (ABd, 1H, *J* = 16.2 Hz), 5.10 (ABd, 1H, *J* = 15.0 Hz), 6.33 (s, 1H), 6.50-6.85 (m, 6H), 7.11-7.14 (m, 2H), 7.39-7.42 (m, 2H), 7.69-7.95 (m, 4H), 8.40 (s, 1H), 8.47 (s, 1H). ¹³C NMR (150 MHz, CD₃OD, a mixture of rotamers): 21.4, 24.3, 24.7, 24.8, 24.9, 25.1, 25.2, 28.9, 32.1, 32.2, 46.0, 49.0, 49.1, 49.3, 52.0, 52.2, 56.5, 111.8, 111.9, 112.4, 113.7, 113.8, 113.9, 114.0, 121.1, 121.2, 121.4, 121.5, 124.8, 125.6, 127.0, 127.1, 128.1, 128.7, 128.8, 130.3, 130.4, 132.9, 133.5, 133.6, 136.3, 160.8, 160.9, 162.4, 162.5, 162.6, 164.8, 165.3, 169.5, 169.6.

3-(2-(*tert*-Butylamino)-1-(*N*-(4-chlorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylic acid (9l): yellow solid (58 mg, 94%). HPLC/MS: t_R = 10.82 min; *m/z* = 476.1 [M+H]⁺. HRMS: $C_{23}H_{23}Cl_2N_3O_4Na$, 498.0963 (calcd.), 498.0947 (found). ¹H NMR (600 MHz, CD₃OD, a mixture of rotamers): 1.25 (s, 9H), 1.31 (s, 5H), 4.29 (m, 2H), 4.70 (ABd, 1H, *J* = 16.2 Hz), 5.13 (ABd, 1H, *J* = 15.0 Hz), 6.25 (s, 1H), 6.47 (m, 1H), 6.77 (s, 1H), 6.81-7.14 (m, 8H), 7.39 (s, 1H), 7.42 (s, 1H), 7.60 (s, 1H), 7.80 (m, 1H), 7.84 (m, 1H), 8.37 (s, 1H), 8.47 (s, 1H). ¹³C NMR (150 MHz, CD₃OD, a mixture of rotamers): 27.4, 27.5, 46.1, 48.4, 51.2, 52.7, 56.9, 111.8, 111.9, 120.9, 121.1, 121.7, 121.9, 124.7, 126.8, 127.2, 127.4, 128.6, 130.3, 130.5, 132.1, 132.2, 135.8, 136.3, 136.5, 165.3, 169.7.

3-(2-(*tert*-Butylamino)-1-(*N*-(3,4-dichlorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylic acid (9m): yellow solid (70 mg, 98%). HPLC/MS: t_R = 11.28 min; *m/z* = 510.2 [M+H]⁺. HRMS: $C_{23}H_{23}Cl_3N_3O_4$, 510.0754 (calcd.), 510.0783 (found). ¹H NMR (600

MHz, CD$_3$OD, a mixture of rotamers): 1.27 (s, 9H), 1.31 (s, 4H), 4.22 (ABd, 1H, J = 15.6 Hz), 4.29 (ABd, 1H, J = 16.8 Hz), 4.70 (ABd, 1H, J = 16.2 Hz), 5.15 (ABd, 1H, J = 16.2 Hz), 5.51 (s, 1H), 6.32 (s, 1H), 6.45 (m, 1H), 6.70-7.15 (m, 6H), 7.40-7.84 (m, 4H), 8.42 (s, 1H), 8.48 (s, 1H). ^{13}C NMR (150 MHz, CD$_3$OD, a mixture of rotamers): 27.4, 27.5, 45.6, 49.1, 51.3, 52.5, 53.5, 56.8, 111.9, 112.0, 112.3, 113.7, 121.1, 121.2, 121.5, 121.7, 124.7, 125.0, 125.4, 126.6, 127.3, 128.3, 128.8, 129.1, 129.2, 129.9, 130.3, 130.4, 130.9, 131.0, 136.1, 136.2, 137.9, 138.5, 162.1, 162.6, 164.7, 165.3, 169.8.

3-(2-(*tert*-Butylamino)-1-(*N*-(2,4-dichlorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylic acid (9n): yellowish solid (74 mg, 85%). HPLC/MS: t_R = 11.34 min; m/z = 509.9 [M+H]$^+$. HRMS: C$_{23}$H$_{22}$Cl$_3$N$_3$O$_4$Na, 532.0574 (calcd.), 532.0572 (found). ^{1}H NMR (600 MHz, CD$_3$OD, a mixture of rotamers): 1.27 (s, 9H), 1.31 (s, 5H), 4.64-4.74 (m, 2H), 4.97-4.99 (m, 2H), 6.38 (s, 1H), 6.82 (s, 1H), 6.99-7.07 (m, 5H), 7.15 (s, 1H), 7.35 (m, 1H), 7.38 (s, 1H), 7.63 (s, 1H), 7.71 (s, 1H), 7.75-7.77 (m, 1H), 7.80 (m, 1H), 8.43 (s, 1H), 8.44 (s, 1H). ^{13}C NMR (150 MHz, CD$_3$OD, a mixture of rotamers): 27.4, 27.5, 44.1, 47.0, 51.3, 52.0, 52.9, 57.0, 111.7, 111.8, 112.1, 113.6, 121.0, 121.2, 121.7, 122.1, 124.5, 125.3, 126.0, 126.1, 127.9, 128.0, 128.7, 129.4, 130.4, 130.5, 132.1, 132.6, 132.8, 134.0, 136.1, 136.3, 165.3, 169.6.

3-(2-(*tert*-Butylamino)-1-(*N*-((6-chloropyridin-3-yl)methyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylic acid (9o): yellow solid (28 mg, yield: 74%). HPLC/MS: t_R = 11.63 min; m/z = 476.5 [M+H]$^+$. HRMS: C$_{22}$H$_{22}$Cl$_2$N$_4$O$_4$Na, 499.0916 (calcd.), 499.0942 (found). ^{1}H NMR (600 MHz, CD$_3$OD, a mixture of rotamers): 1.16 (s, 9H), 1.19 (s, 4H), 4.21 (ABd, 1H, J = 15.6 Hz), 4.27 (ABd, 1H, J = 16.8 Hz), 4.62 (ABd, 1H, J = 16.8 Hz), 5.02 (ABd, 1H, J = 15.6 Hz), 6.16 (s, 1H), 6.61 (s, 1H), 6.92 (m, 1H), 6.97-7.04 (m, 2H), 7.16 (m, 2H), 7.29 (s, 1H), 7.32 (s, 1H), 7.51 (s, 1H), 7.56 (s, 1H), 7.68-7.72 (m, 2H), 8.27 (s, 1H), 8.41 (s, 1H). ^{13}C

NMR (150 MHz, CD$_3$OD, a mixture of rotamers): 28.8, 28.9, 45.0, 52.7, 54.0, 58.3, 113.4, 113.5, 115.3, 122.7, 122.8, 123.0, 123.3, 124.4, 124.6, 125.9, 132.0, 132.1, 134.2, 134.6, 137.6, 138.3, 139.9, 147.6, 149.1, 150.5, 166.8, 171.0.

3-(2-(*tert*-Butylamino)-1-(*N*-((*S*)-1-(4-chlorophenyl)ethyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylic acid (9p): yellow solid (46 mg, 92%). HPLC/MS: t_R = 9.30 min; *m/z* = 490.0 [M+H]$^+$. HRMS: C$_{24}$H$_{25}$Cl$_2$N$_3$O$_4$Na, 512.1120 (calcd.), 512.1072 (found). ^{1}H NMR (400 MHz, CD$_3$OD, a 3:2 mixture of diastereomers): 1.02 (s, 9H), 1.29 (s, 7H), 1.65 (d, 3H, *J* = 7.2 Hz), 1.75 (d, 2H, *J* = 7.2 Hz), 5.14 (q, 1H, *J* = 6.8 Hz), 5.75 (q, 1H, *J* = 6.8 Hz), 6.25 (s, 1H), 6.70 (s, 1H), 6.81 (m, 1H), 6.98-7.06 (m, 5H), 7.14-7.20 (m, 2H), 7.34-7.53 (m, 7H), 7.72-7.86 (m, 2H), 8.40 (s, 1H), 8.45 (s, 1H). ^{13}C NMR (100 MHz, CD$_3$OD, a mixture of diastereomers): 16.2, 17.6, 21.3, 27.3, 27.4, 27.5, 50.2, 50.5, 50.7, 50.8, 52.8, 54.3, 54.7, 56.3, 108.2, 109.8, 110.8, 111.5, 111.6, 120.1, 120.3, 121.4, 121.6, 125.3, 126.8, 127.5, 127.7, 128.1, 128.7, 130.8, 131.8, 133.3, 134.3, 135.3, 137.3, 140.9, 141.8, 163.5, 165.6, 165.7, 168.1, 169.2, 170.5.

3-(2-(*tert*-Butylamino)-1-(*N*-((*R*)-1-(4-chlorophenyl)ethyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylic acid (9q): yellow solid (68 mg, 98%). HPLC/MS: t_R = 11.28 min; *m/z* = 490.2 [M+H]$^+$. HRMS: C$_{24}$H$_{26}$Cl$_2$N$_3$O$_4$, 490.1300 (calcd.), 490.1290 (found). ^{1}H NMR (400 MHz, CD$_3$OD, a 3:2 mixture of diastereomers): 1.02 (s, 9H), 1.27 (s, 6H), 1.64 (d, 3H, *J* = 6.8 Hz), 1.76 (m, 2H), 5.12 (m, 1H), 5.75 (q, 1H, *J* = 6.8 Hz), 6.26 (s, 1H), 6.69 (s, 1H), 6.75 (m, 1H), 6.95-7.18 (m, 5H), 7.34-7.52 (m, 6H), 7.72-7.83 (m, 2H), 8.42 (m, 1H), 8.45 (s, 1H). ^{13}C NMR (100 MHz, CD$_3$OD, a mixture of diastereomers): 16.2, 17.6, 21.6, 27.3, 27.4, 27.5, 29.4, 50.8, 50.9, 54.7, 56.4, 110.9, 111.5, 111.6, 120.4, 121.4, 125.4, 126.6, 127.5, 127.6, 128.1, 128.8, 129.3, 130.8, 132.0, 133.3, 134.2, 135.3, 137.3, 140.8, 141.7, 165.6, 169.2, 170.4.

3-(2-(*tert*-Butylamino)-1-(*N*-(4-hydroxybenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylic acid (9r): yellow solid (24 mg, 91%). HPLC/MS: t_R = 10.65 min; *m/z* = 458.2 [M+H]$^+$. HRMS: $C_{23}H_{24}ClN_3O_5Na$, 480.1302 (calcd.), 480.1336 (found). ^{1}H NMR (600 MHz, CD$_3$OD, a mixture of rotamers): 1.21 (s, 9H), 1.31 (s, 7H), 4.21-4.28 (m, 2H), 4.59 (ABd, 1H, *J* = 16.2 Hz), 5.06 (ABd, 1H, *J* = 14.4 Hz), 6.17 (s, 1H), 6.34-6.39 (m, 2H), 6.54 (m, 2H), 6.75 (s, 1H), 6.83 (m, 2H), 7.09-7.13 (m, 2H), 7.41-7.50 (m, 3H), 7.79 (m, 1H), 7.84 (m, 1H), 8.31 (s, 1H), 8.41 (s, 1H). ^{13}C NMR (150 MHz, CD$_3$OD, a mixture of rotamers): 27.4, 27.5, 46.6, 49.6, 51.0, 51.2, 52.0, 52.9, 56.8, 111.8, 111.9, 112.8, 114.1, 114.3, 114.6, 120.8, 121.1, 121.9, 122.1, 124.8, 125.6, 126.7, 127.4, 127.5, 128.2, 129.1, 130.2, 130.5, 136.4, 156.0, 156.2, 162.3, 164.6, 165.2, 169.5.

3-(1-(*N*-(Biphenyl-4-ylmethyl)formamido)-2-(*tert*-butylamino)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylic acid (9s): yellow solid (70 mg, 89%). HPLC/MS: t_R = 11.50 min; *m/z* = 518.1 [M+H]$^+$. HRMS: $C_{29}H_{29}ClN_3O_4$, 518.1847 (calcd.), 518.1844 (found). ^{1}H NMR (600 MHz, CD$_3$OD, a mixture of rotamers): 1.21 (s, 9H), 1.31 (s, 5H), 4.33-4.37 (m, 2H), 4.74 (ABd, 1H, *J* = 16.2 Hz), 5.22 (ABd, 1H, *J* = 15.0 Hz), 6.30 (s, 1H), 6.55 (m, 1H), 6.81 (s, 1H), 6.98-7.16 (m, 5H), 7.29-7.56 (m, 14H), 7.81 (m, 1H), 7.87 (m, 1H), 8.41 (s, 1H), 8.49 (s, 1H). ^{13}C NMR (150 MHz, CD$_3$OD, a mixture of rotamers): 27.4, 27.6, 46.7, 49.9, 51.1, 51.2, 51.3, 52.0, 52.9, 56.9, 111.7, 111.9, 112.5, 114.1, 120.9, 121.1, 121.8, 122.0, 124.8, 125.6, 125.8, 126.0, 126.5, 126.8, 127.9, 128.3, 128.4, 130.3, 130.4, 135.8, 136.3, 136.6, 139.6, 139.7, 140.7, 164.7, 165.3, 169.6, 169.9.

3-(2-(*tert*-Butylamino)-2-oxo-1-(*N*-(4-phenoxybenzyl)formamido)ethyl)-6-chloro-1*H*-indole-2-carboxylic acid (9t): yellow solid (68 mg, 92%). HPLC/MS: t_R = 11.53 min; *m/z* = 534.3 [M+H]$^+$. HRMS: $C_{29}H_{28}ClN_3O_5Na$, 556.1615 (calcd.), 556.1614 (found). ^{1}H NMR (600 MHz,

CD$_3$OD, a mixture of rotamers): 1.26 (s, 9H), 1.32 (s, 6H), 4.28-4.32 (m, 2H), 4.70 (ABd, 1H, J = 16.2 Hz), 5.17 (ABd, 1H, J = 15.6 Hz), 6.30 (s, 1H), 6.44-6.90 (m, 10H), 7.09-7.44 (m, 8H), 7.59-7.87 (m, 4H), 8.39 (s, 1H), 8.48 (s, 1H). ^{13}C NMR (150 MHz, CD$_3$OD, a mixture of rotamers): 27.45, 27.51, 46.2, 51.20, 51.25, 56.8, 111.8, 111.9, 112.5, 114.0, 117.7, 117.8, 118.1, 118.2, 120.9, 121.0, 121.8, 122.0, 122.7, 122.8, 124.8, 125.6, 126.7, 128.6, 129.4, 129.5, 130.2, 130.3, 131.9, 132.6, 136.2, 136.3, 155.9, 156.0, 157.3, 157.4, 164.6, 165.3, 169.8, 170.0.

Synthesis of ethyl 3-(1-(benzylamino)-2-(cyclohexylamino)-2-oxoethyl)-6-chloro-1H-indole-2-carboxylate (10): 7i was treated with 0.4 mL of dioxane (2M HCl), 0.1 mL of water stirring overnight under RT. After quenched by 0.5 mL of triethyl amine, the product was purified by chromatography on silica gel (petroleum ether/ethyl acetate, 1:2) as yellowish solid (34 mg, yield: 58%). HRMS: C$_{26}$H$_{30}$ClN$_3$O$_3$Na, 490.1873 (calcd.), 490.1864 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 0.82-1.06 (m, 6H), 1.26-1.37 (m, 10H), 1.59-1.92 (m, 10H), 3.64-3.83 (m, 6H), 4.30-4.70 (m, 7H), 5.61-5.74 (m, 2H), 6.20 (s, 1H), 6.60 (m, 2H), 6.78 (s, 1H), 6.97-7.27 (m, 12H), 7.50 (m, 1H), 7.78 (m, 1H), 8.44 (s, 1H), 8.53 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.3, 14.4, 24.76, 24.80, 24.9, 25.3, 25.4, 32.6, 32.7, 32.8, 32.9, 42.9, 46.4, 48.88, 48.92, 50.1, 52.3, 57.3, 61.6, 61.65, 61.68, 71.1, 72.3, 111.9, 112.3, 113.5, 115.1, 122.2, 122.3, 122.4, 122.7, 124.8, 125.6, 126.0, 126.3, 127.2, 127.3, 127.8, 127.9, 128.2, 128.4, 128.5, 131.6, 131.8, 131.9, 136.0, 137.3, 137.4, 160.6, 160.7, 163.7, 164.8, 167.9, 168.1.

Synthesis of 3-(1-(benzylamino)-2-(cyclohexylamino)-2-oxoethyl)-6-chloro-1H-indole-2-carboxylic acid (11a): A mixture of **10** (28 mg), EtOH (0.5 mL), water (0.5 mL), and KOH (10 mg) was heated to reflux for 5 h. After cooling, the reaction mixture was acidified with 1M HCl to pH ~ 6. The mixture was then extracted with DCM (10 mL x 3). The combined organic layer

was dried over sodium sulfate. After evaporation of the solvent, 21 mg of yellow solid (74%) was obtained. HPLC/MS: t_R = 12.10 min; m/z = 440.4 [M+H]$^+$. HRMS: $C_{24}H_{27}N_3O_3Cl$, 440.1741 (calcd.), 440.1721 (found). ^{1}H NMR (600 MHz, d^6-DMSO, a mixture of rotamers): 0.85-1.63 (m, 16H), 3.57 (m, 2H), 4.17 (ABd, 1H, J = 16.2 Hz), 4.22 (ABd, 1H, J = 15.6 Hz), 4.67 (ABd, 1H, J = 16.8 Hz), 4.88 (ABd, 1H, J = 15.6 Hz), 6.10 (s, 1H), 6.51 (m, 1H), 6.60 (s, 1H), 6.78 (m, 2H), 6.91-7.28 (m, 7H), 7.66-7.91 (m, 4H), 8.27 (s, 1H), 8.34 (s, 1H), 11.56 (s, 1H), 11.88 (s, 1H), 13.15 (br.s, 1H), 13.53 (br.s, 1H). ^{13}C NMR (150 MHz, d^6-DMSO, a mixture of rotamers): 25.0, 25.1, 25.5, 32.5, 32.6, 47.0, 48.5, 56.2, 112.4, 121.3, 122.4, 125.2, 125.8, 126.7, 126.8, 127.1, 127.7, 127.9, 129.0, 129.3, 136.4, 138.0, 164.2, 168.8.

Synthesis of 3-(2-(tert-butylamino)-1-(4-chlorobenzylamino)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylic acid (11b): Formamide **9l** (24 mg, 0.05 mmol) was treated with 0.4 mL of dioxane (2M HCl) and 0.1 mL of water stirred overnight at 60 °C. After evaporation, the product was obtained as a yellow solid (20 mg, yield: 89%). HPLC/MS: t_R = 10.42 min; m/z = 448.3 [M+H]$^+$. HRMS: $C_{22}H_{22}Cl_2N_3O_3$, [M-H]$^-$; 446.1038 (calcd.), 446.1050 (found). ^{1}H NMR (600 MHz, CD$_3$OD, a mixture of rotamers): 1.25 (s, 9H), 1.31 (s, 6H), 4.27-4.33 (m, 2H), 4.70 (ABd, 1H, J = 16.2 Hz), 5.13 (ABd, 1H, J = 15.6 Hz), 5.88 (s, 1H), 6.24 (s, 1H), 6.47 (m, 1H), 6.76-7.27 (m, 8H), 7.39-7.60 (m, 6H), 7.79-7.85 (m, 2H), 8.38 (s, 1H), 8.47 (s, 1H), 11.35 (s, 1H), 11.60 (s, 1H). ^{13}C NMR (150 MHz, CD$_3$OD, a mixture of rotamers): 27.3, 27.4, 46.5, 50.9, 51.0, 57.1, 111.6, 120.4, 121.3, 125.0, 126.8, 127.3, 127.5, 127.9, 128.6, 128.9, 129.0, 129.6, 131.0, 132.1, 135.3, 135.5, 170.1.

Synthesis of 3-(1-(*N*-benzylacetamido)-2-(cyclohexylamino)-2-oxoethyl)-6-chloro-*N*-(2-(pyridin-4-yl)ethyl)-1*H*-indole-2-carboxamide (13a): The mixture of **8e** (50.9 mg, 0.1 mmol), THF (1 mL), 4-(2-aminoethyl)pyridine (0.2 mmol, 23.7 µL), and TBD (0.02 mmol, 3 mg) was

stirred at 40 $^{\circ}$C overnight. The product was purified by chromatography on silica gel (methanol/ethyl acetate, 1:5) as yellow solid (25 mg, yield: 43%). HPLC/MS: t_R = 9.24 min; m/z = 586.0 [M+H]$^+$. HRMS: $C_{33}H_{36}ClN_5O_3Na$, 608.2404 (calcd.), 608.2427 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.85-1.34 (m, 6H), 1.57-1.92 (m, 7H), 2.03 (s, 3H), 3.04 (m, 2H), 3.74-3.90 (m, 3H), 4.75-4.90 (m, 2H), 5.80 (s, 1H), 6.52 (m, 2H), 6.86 (s, 1H), 6.95-7.02 (m, 4H), 7.20 (m, 2H), 7.32 (s, 1H), 7.49 (m, 1H), 8.42 (m, 2H), 8.57 (s, 1H), 10.11 (br.s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 22.2, 24.7, 24.8, 24.9, 25.3, 29.7, 32.7, 33.0, 34.4, 40.4, 49.3, 51.5, 53.5, 108.3, 112.1, 121.5, 122.1, 124.2, 125.1, 125.2, 126.9, 128.2, 130.6, 131.3, 135.3, 136.9, 148.1, 149.7, 150.3, 160.8, 167.9, 174.3.

Synthesis of 3-(1-(*N*-benzylacetamido)-2-(cyclohexylamino)-2-oxoethyl)-6-chloro-*N*-(2-methoxyethyl)-1*H*-indole-2-carboxamide (13b): A mixture of **8e** (50.9 mg, 0.1 mmol), THF (1 mL), 2-methoxyethylamine (0.2 mmol, 17.5 µL), and TBD (0.02 mmol, 3 mg) was stirred at 40 $^{\circ}$C overnight. The product was purified by chromatography on silica gel (ethyl acetate) as yellowish solid (14 mg, yield: 26%). HPLC/MS: t_R = 15.45 min; m/z = 539.4 [M+H]$^+$. HRMS: $C_{29}H_{35}N_4O_4Cl$, 538.234684 (calcd.), 538.234872 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.85-1.33 (m, 7H), 1.56-1.92 (m, 7H), 2.18 (s, 3H), 3.36-3.83 (m, 8H), 4.68 (ABd, 1H, J = 18.0 Hz), 4.92 (ABd, 1H, J = 17.4 Hz), 6.65 (m, 2H), 6.84 (s, 1H), 6.97 (m, 3H), 7.06 (m, 1H), 7.59 (m, 1H), 7.94 (s, 1H), 10.03 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 22.2, 24.8, 24.9, 25.3, 32.7, 32.9, 39.5, 40.0, 49.2, 51.2, 53.8, 58.7, 58.8, 58.9, 70.5, 70.6, 108.4, 112.1, 112.2, 120.8, 121.5, 121.6, 122.0, 125.3, 126.8, 127.6, 127.9, 128.1, 128.8, 130.5, 131.6, 135.4, 137.3, 159.8, 161.0, 168.2, 173.8.

2.3.2   Exhaustive fluorine scanning toward potent p53-Mdm2 inhibitors*

The unmatched properties of the element fluorine are reflected in organofluorine compounds. The richness of modern fluorine chemistry allows for the regioselective introduction of this element at virtually every position of a given molecule.[135, 136] Thus, the introduction of fluorine has been especially valuable in the process of drug discovery to fine tune many different target and off-target related properties.[137-141] For example, fluorine has been used to increase the binding affinity of small molecules to its target,[142] to tune the pK_B and logD,[143] to improve target selectivity,[144] to improve oral absorption and exposure,[145] to prevent metabolism[146] or to increase the antibacterial spectrum.[147] Not surprisingly, an estimated 20% of all pharmaceuticals contain fluorine.[139] Additionally, ^{18}F is often used as a positron emission tomography (PET) active element to perform time- and space- resolved distribution studies of drugs in animals and humans.[148, 149] Last but not least, ^{19}F is very useful in NMR analysis of complex biological systems due to its high sensitivity and zero natural background.[150] Herein is reported the systematic F-scanning of a scaffold derived from the Ugi-4CR for the synthesis, optimization and biophysical characterization of potent p53-Mdm2 inhibitors.

Due to the wealth of structural information available for the p53-Mdm2 interaction,[85, 88] a pharmacophore-based virtual screening platform ANCHOR.QUERY was recently introduced for the rational design of small molecule inhibitors.[151] Based on this approach we described a number of unprecedented MCR scaffolds, which are able to efficiently disrupt the p53-Mdm2 interaction.[84, 92, 123, 125] The structurally characterized p53-Mdm2 inhibitor **9l** showed a nice alignment with the three-finger pharmacophore model for Mdm2 inhibitors,[152] in which key

moieties fit into the Trp23, Phe19 and Leu26 binding pockets.[153] Notably, the benzyl substituent of the small molecule is involved in an stacking interaction with the imidazole ring of His96 (**Figure 14**).[152] In an attempt to modulate the π-stacking interaction, all 19 isomers with different fluorine substitution pattern possible for the benzyl position were synthesized (**Scheme 1**). Fluorine-substituted benzylamines **14b-t** were obtained from commercial sources or prepared from the corresponding benzylaldehydes by reductive amination.[154] The compounds **17a-t** were synthesized by the Ugi-4CR in good to excellent yields. The ester group of **17a-t** was saponified to give the corresponding acid compounds **18a-t**, since the 2-carboxylic acid of the indole ring is known to improve the binding affinity with Mdm2.[84, 123, 125] By the nature of the Ugi reaction, all products were formed as racemic mixtures and screened as such.
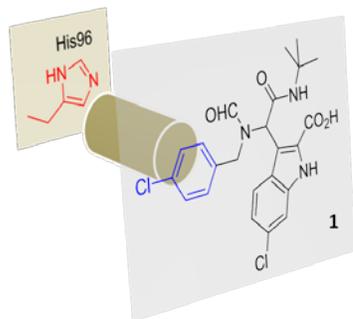
Figure 14. A co-crystal structure of p53-Mdm2 inhibitor (*S*)-**9l** reveals a parallel alignment between the benzyl group of the Ugi scaffold and the imidazole ring of His96 (Mdm2), which seems suitable for optimization of the π-π interaction.
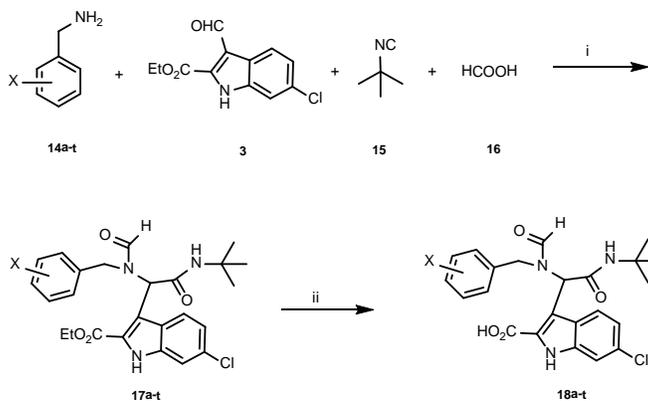
Scheme 6. Synthesis of p53-Mdm2 inhibitors using the Ugi-4CR followed by saponification. Conditions: (i) MeOH, r.t.; (ii) LiOH, EtOH/H$_2$O (1:1), r.t.

The K_i values of the compounds **17** and **18** to Mdm2 were measured using the FP assay (**Table 1**).[155] The well known nutlin-3a was used as a reference compound and the binding data well compare with previously published results.[92] The acid compounds **18** showed overall improved potency compared with the corresponding parent ethyl ester compounds **17**. Interestingly, the K_i values of compounds **18** with different fluorine substitution patterns varied by a factor of 44, between 5.7 µM and 130 nM. The best compound, **18m** (K_i = 130 nM, molecular weight: 495 Da), is amongst the most potent p53-Mdm2 inhibitors known, and has comparable affinity to nutlin-3a (K_i = 40 nM, molecular weight: 581.5 Da.). Compound **18m** exhibits a BEI of 13.9, which indicates a better ligand efficiency than nutlin-3a (BEI = 12.7).[156] The aqueous solubility and calculated lipophilicity of **18m** is 0.85 mg/mL and cLogP = 3.69 (SI), which is superior compared to nutlin-3a (0.1 mg/mL, cLogP = 5.17).

Table 6. Inhibition constants [µM] of compounds **17** and **18**.

No.	X	K_i (**17**) [a]	K_i (**18**) [a]	cLogP (**18**) [b]
a	H	1.5	1.8	3.26
b	4-F	2.2	0.45	
c	3-F	1.3	0.81	3.40
d	2-F	3	1.7	
e	3,4-F	0.5	0.25	
f	2,4-F	6	2.3	
g	2,3-F	3	0.2	3.54
h	2,5-F	10	2.5	
i	3,5-F	2.4	0.3	
j	2,6-F	4.5	5.7	

k	2,3,4-F	2.1	0.15	
l	2,4,5-F	5	2.3	
m	3,4,5-F	0.4	0.13	3.69
n	2,3,6-F	4.3	3.2	
o	2,4,6-F	6.8	3.2	
p	2,3,5-F	2.5	0.17	
q	2,3,5,6-F	6.7	3	
r	2,3,4,6-F	5.8	3	3.83
s	2,3,4,5-F	7	0.7	
t	2,3,4,5,6-F	5.8	1.8	3.97
Nutlin-3a			0.04	5.17

[a]Measured by fluorescent polarization assay. [b]Calculated using Instant JChem 2.5.3 from ChemAxon.

In order to better understand the structural basis of the interaction, **18e** was co-crystallized with Mdm2 and the X-ray structure was resolved (**Figure 15**). As expected, the small molecule binds into the p53 binding site of Mdm2. The 6-chloro-indole moiety aligns well with the anchor residue Trp23 of p53 and forms a hydrogen bond to Leu54. The 3,4-difluorobenzyl group mimics the Leu26 and the *tert*-butylamide substituent derived from the isocyanide component is deeply buried in the Phe19 pocket. The formyl substituent points into the solvent space. Two amide oxygen atoms of **18e** and the residues in the receptor (His96 and Val93) form hydrogen bond bridges with surrounding water molecules. The overall interactions, however, are mostly governed by hydrophobic contacts involving the indole, 3,4-difluorobenzyl and *tert*-butyl substituent and receptor amino acids. Clearly, the 3,4-difluorobenzyl group is

nicely aligned parallel with the imidazole ring of His96 suggesting an attractive interaction. Short distances between the atoms of two aromatic rings are between 3.4-3.9 Å. Compared to other crystallographically characterized small molecule inhibitors of the p53-Mdm2 interaction,[85, 88] the Leu26 binding site encloses the 3,4-difluorobenzyl group very tightly.
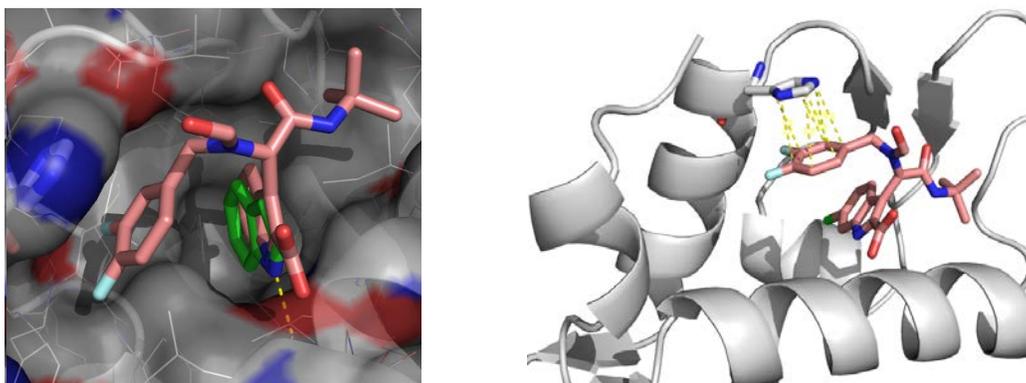
Figure 15. The cocrystal structure of compound (*S*)-**18e** (pink sticks) in Mdm2 (surface with cartoon secondary structure and lines). His96, forming a π-stacking interaction with the 3,4-difluoro benzyl group of (*S*)-**18e** is highlighted as blue sticks. The indol fragment of (*S*)-**18e** aligns well with the anchor fragment Trp23 of p53 (green sticks, PDB ID: 1YCR; RMSD = 0.701 Å). The indol NH of (*S*)-**18e** also forms the characteristic conserved hydrogen bond with the carbonyl group of Leu54 (yellow dotted line). The structural data is available from the PDB (ID: 3TU1, resolution: 1.6 Å).
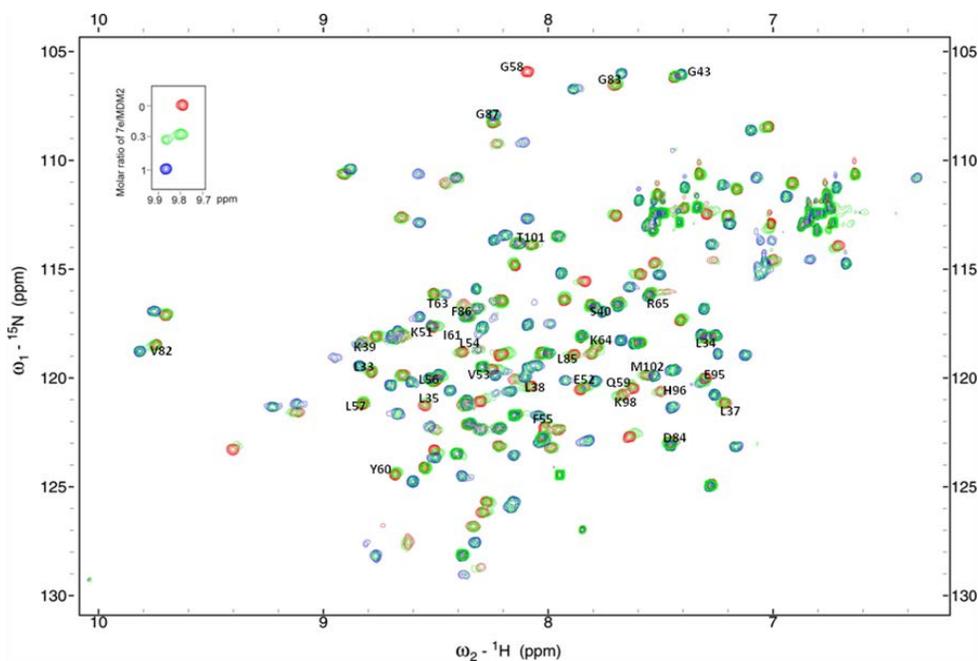
Figure 16. Superposition of NMR HSQC spectra of ^{15}N-labeled Mdm2 titrated against **(+)-18e**. The spectrum of free Mdm2 is shown in red. The spectrum of **(+)-18e**-Mdm2 (intermediate ratio, 3:10, respectively) is shown in green, and the spectrum of **(+)-18e**-Mdm2 (final ratio, 1:1) is shown in blue.

The enantiomers of **17e** and **17m** were efficiently separated by preparative SFC, and transformed to the corresponding enantiomers of **18e** and **18m**, respectively (**Appendix B**). The enantiomer **(+)-18e** (K_i = 200 nM) was more potent than **(-)-18e** (K_i = 400 nM). Similarly, the enantiomer **(+)-18m** (K_i = 100 nM) was more potent than **(-)-18m** (K_i = 280 nM). **Figure 16** shows a NMR HSQC experiment for **(+)-18e** and Mdm2. A strong binding, with K_D of less than 1 μM, (and a slow chemical exchange) of **(+)-18e** to Mdm2 is indicated by the signal doubling.[133, 134]

A key property of the C-F bond is the reversed polarization as compared to the C-H bond. Thus, the pattern of fluorine substitutions on the benzene ring should be able to modulate the aromatic interaction between the small molecule and its protein receptor.[157, 158] This effect was studied using docking models derived from the co-crystal structure (**Figure 2**) by performing all possible fluorine substitution patterns in the benzyl group of compounds **18b-t** on a fixed receptor. All the refined docked small molecules showed only minor differences relative to the co-crystal structure. The models reveal that the C-H to C-F substitution at the buried *ortho*-position potentially leads to a highly repulsive dipole-dipole interaction (**Figure 4**). In fact, the straightforward change in Coulomb energy due to the charge swap indicated in **Fig. 4** is about 4 kcal/mol. Not surprisingly, the fluorine in the buried *ortho*-position does not rank well either in our models or in the experiments. Note that, at least for the subset of compounds with symmetric fluorine patterns, there is no ambiguity on the position of the fluorines in the docked models based on the co-crystal structure.

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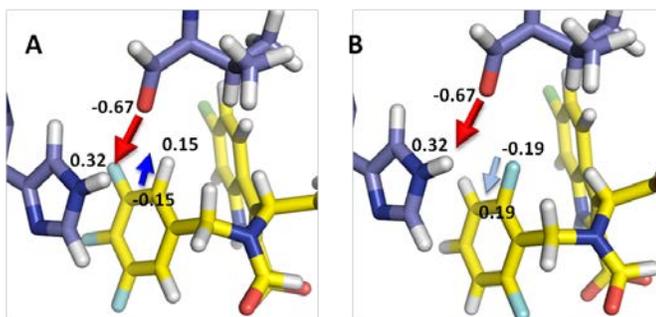
Figure 17. Dipole reversal by fluorine is the main determinant of binding affinity. Docking models (yellow sticks) of (A) compound **(S)-18m** and (B) compound **(S)-18j** are minimized structures of the superposition of the small molecules onto the ligand of the co-crystal shown in Fig. 2 with a fixed receptor. Arrows depict the dipole-dipole interaction between the His96.ε-Val93.O hydrogen bond (blue sticks, red arrow) and the (A) C-H (blue arrow) and (B) C-F (cyan arrow) dipoles that are attractive and repulsive, respectively. Also indicated are the MMFF (Merck Molecular Force Field) partial charges.
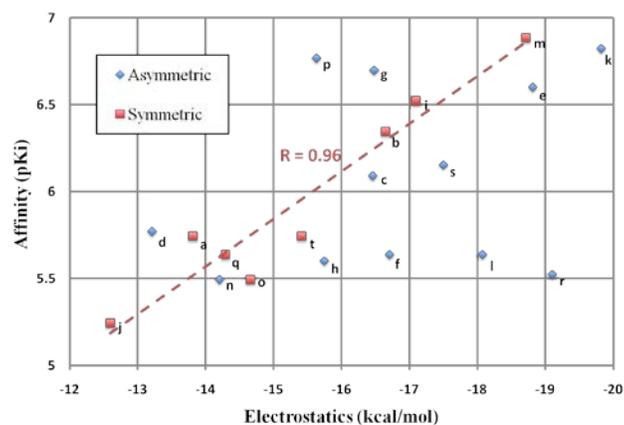
Figure 18. Correlation between the calculated change in electrostatics and binding affinity (pKi) for compounds (S)-18. Electrostatic calculations were performed using OpenEye Szybki 1.3.1 with a Poisson-Boltzmann solvation model and MMFF partial charges. Compounds with a symmetric arrangement of fluorine atoms in the benzyl group correlate closely (R = 0.96) to the calculated electrostatics. The remaining asymmetric compounds are more difficult to characterize and reduce the overall correlation (R = 0.58). Following Table 1, letters indicate the fluorine substitution pattern in the phenyl ring.

Given the strong electrostatic coupling between the two rings, it was expected that the inter-molecular component of the electrostatic free energy would be the main determinant of binding affinity. Indeed, as shown in **Figure 18**, the correlation coefficient between the computed change in electrostatics and the experimentally determined affinity (pKi) yielded a strikingly accurate correlation (R = 0.96) for compounds with symmetric fluorine substitutions.

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The analysis correctly ranks compounds with the best and worst Ki values among the benzyl rings with symmetric F-pattern. By itself, this result is quite impressive, since most physically based scoring functions do not recapitulate thermodynamic data.[159] The correlation of electrostatics with pKi for the full set of compounds, including those with fluorine substitutions that form asymmetric patterns, sharply dropped the correlation (R = 0.58). An even lower correlation (R = 0.22) was obtained using the full binding affinities calculated by the software OpenEye Szybki, further emphasizing the key role of electrostatics in capturing changes in Ki for the different ligands. Refining the ligand protonation states by considering pKa and tautomer enumeration with the AM1-BCC charge model of OpenEye QuacPac version 1.3.1 do not change results.[160] The conspicuous over-prediction of the electrostatic component of the binding free energy for asymmetric compounds is likely to reflect the specificities of the internal energies for each rotamer of the benzyl ring. This effect is offset for symmetric compounds, but it should significantly affect the free energy of the unbound asymmetric ligands in ways that are ignored by the fixed ligand structures imposed by the Poisson-Boltzmann calculations.

In summary, 20 derivatives of p53-Mdm2 inhibitor **9l** were efficiently synthesized via a one-pot Ugi-4CR including all possible fluorine substitutions on the benzyl group in order to optimize aromatic interactions. A co-crystal structure of (**S**)**-18e** with Mdm2 reveals the structural basis of the potent interaction. The introduction of fluorine substitutions on the benzyl group can considerably improve the potency of p53-Mdm2 inhibitors, due to the electrostatic interaction between the small molecules and the receptor. Although limited in scope, the computational analysis of docked configurations that are presumed to bind using the same binding mode shows that the electrostatics of unique rigid structures can be reasonably be accounted for by current methods, whereas assessing the free energy of an unbound state with a

variety of hard to evaluate internal states is quite detrimental to empirical estimates of the free energy. These findings underscore the in principal known, but often surprising, effects that fluorine can exert on the biological activity of small molecules. These findings may also lead medicinal chemists to success in modulating molecular interactions with other types of targets.

Materials and Methods

Fluorine substituted benzylamines **14** were purchased or prepared from the corresponding benzylaldehydes according to the literature.[161] The purification was conducted using preparative silica gel TLC plates (1000 μm, 20cm×20cm).

General procedure for the synthesis of ester compounds: The mixture of benzyl amine (**14**, 0.2 mmol), aldehyde (**3**, 0.2 mmol), *tert*-butyl isocyanide (**15**, 0.2 mmol), and formic acid (**16**, 0.2 mmol) in 0.5 mL of methanol was stirred at RT for 5-7 days. The product (**17a-t**) was purified by chromatography on silica gel (petroleum ether/ethyl acetate, 1:1).

Ethyl 3-(1-(*N*-benzylformamido)-2-(*tert*-butylamino)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (17a): yellow solid (51 mg, yield: 55%). HPLC/MS: t_R = 10.89 min; m/z = 469.5 [M+H]$^+$. HRMS: $C_{25}H_{28}ClN_3O_4Na$, 492.1666 (calcd.), 492.1654 (found). ^{1}H NMR (400 MHz, CDCl$_3$, a mixture of rotamers): 1.22 (s, 9H), 1.31 (s, 7H), 1.38-1.41 (m, 6H), 4.37-4.51 (m, 6H), 4.62 (ABd, 1H, J = 16.0 Hz), 4.84 (ABd, 1H, J = 15.2 Hz), 5.50-5.57 (m, 2H), 6.13 (s, 1H), 6.56 (m, 2H), 6.72 (s, 1H), 6.94-7.05 (m, 4H), 7.14-7.18 (m, 5H), 7.30-7.35 (m, 6H), 7.63 (d, 1H, J = 8.8 Hz), 7.89 (d, 1H, J = 8.8 Hz), 8.04 (s, 1H), 8.28 (m, 1H), 8.44 (s, 1H), 8.52 (s, 1H), 9.31 (s, 1H), 9.65 (s, 1H). ^{13}C NMR (100 MHz, CDCl$_3$, a mixture of rotamers): 14.4, 28.4, 28.6, 28.9, 30.9, 42.2, 46.6, 50.1, 51.9, 52.6, 57.6, 61.6, 111.8, 112.2, 113.5, 115.6, 122.3, 122.5, 122.9,

124.9, 125.7, 125.8, 126.3, 127.1, 127.3, 127.7, 127.8, 127.9, 128.3, 128.8, 129.5, 131.6, 131.9, 135.9, 136.0, 137.4, 137.5, 160.6, 160.7, 161.0, 162.9, 163.6, 164.6, 168.0, 168.1.

Ethyl 3-(2-(*tert*-butylamino)-1-(*N*-(4-fluorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (17b): yellowish solid (75 mg, yield: 77%). HPLC/MS: t_R = 11.37 min; m/z = 488.2 [M+H]$^+$. HRMS: C$_{25}$H$_{27}$ClFN$_3$O$_4$Na, 510.1572 (calcd.), 510.1576 (found). ^{1}H NMR (400 MHz, CDCl$_3$, a mixture of rotamers): 1.25 (s, 9H), 1.29 (s, 6H), 1.34-1.38 (m, 6H), 4.23-4.33 (m, 5H), 4.57 (ABd, 1H, J = 16.0 Hz), 4.91 (ABd, 1H, J = 15.2 Hz), 5.60 (m, 2H), 6.12 (s, 1H), 6.50 (m, 1H), 6.61 (m, 1H), 6.73-6.77 (m, 3H), 6.88-6.91 (m, 2H), 7.11-7.16 (m, 2H), 7.31 (m, 2H), 7.65 (d, 1H, J = 8.8 Hz), 7.86 (d, 1H, J = 8.8 Hz), 8.41 (s, 1H), 8.46 (s, 1H). ^{13}C NMR (100 MHz, CDCl$_3$, a mixture of rotamers): 14.31, 14.34, 28.4, 28.6, 28.9, 30.8, 46.0, 49.4, 51.9, 52.0, 52.6, 57.2, 61.6, 122.1, 122.4, 113.4, 114.4, 114.6, 114.7, 114.9, 115.1, 122.2, 122.3, 122.5, 122.7, 124.7, 125.5, 126.3, 127.3, 127.4, 127.5, 129.3, 131.6, 131.8, 133.1, 133.2, 133.3, 136.1, 136.2, 160.6, 160.7, 163.6, 164.6, 168.1, 168.2.

Ethyl 3-(2-(*tert*-butylamino)-1-(*N*-(3-fluorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (17c): yellow solid (79 mg, yield: 81%). HRMS: C$_{25}$H$_{27}$ClFN$_3$O$_4$Na, 510.1572 (calcd.), 510.1600 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.26 (s, 9H), 1.30 (s, 6H), 1.36-1.38 (m, 6H), 4.26-4.35 (m, 5H), 4.60 (ABd, 1H, J = 16.8 Hz), 4.93 (ABd, 1H, J = 15.6 Hz), 5.65 (m, 2H), 6.16 (s, 1H), 6.21-6.28 (m, 2H), 6.62 (m, 2H), 6.65-6.78 (m, 3H), 6.86 (m, 1H), 6.99-7.03 (m, 2H), 7.13-7.16 (m, 2H), 7.32 (m, 2H), 7.65 (d, 1H, J = 9.0 Hz), 7.86 (d, 1H, J = 8.4 Hz), 8.42 (s, 1H), 8.48 (s, 1H), 9.86 (s, 1H), 10.15 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.3, 14.4, 28.4, 28.6, 28.9, 30.9, 46.2, 49.6, 50.5, 50.7, 51.4, 51.9, 52.1, 52.5, 57.2, 61.7, 112.1, 112.4, 112.5, 112.6, 112.9, 113.8, 113.9, 114.2, 114.3, 114.9, 121.3, 122.1, 122.4, 122.5, 123.0, 123.2, 124.7, 125.5, 126.4, 127.4, 129.2, 129.3,

129.5, 131.6, 131.8, 136.1, 139.9, 140.3, 160.8, 160.9, 161.5, 161.7, 163.2, 163.3, 163.6, 164.7, 168.2.

Ethyl 3-(2-(*tert*-butylamino)-1-(*N*-(2-fluorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (17d): yellow solid (82 mg, yield: 84%). HRMS: $C_{25}H_{27}ClFN_3O_4Na$, 510.1572 (calcd.), 510.1604 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.24 (s, 9H), 1.31 (s, 7H), 1.34-1.37 (m, 6H), 4.28-4.34 (m, 4H), 4.44 (ABd, 1H, J = 16.2 Hz), 4.54 (ABd, 1H, J = 15.6 Hz), 4.60 (ABd, 1H, J = 16.2 Hz), 5.02 (ABd, 1H, J = 15.0 Hz), 5.67 (m, 2H), 6.15 (s, 1H), 6.38 (m, 1H), 6.67 (s, 1H), 6.69 (m, 1H), 6.76 (m, 1H), 6.87 (m, 1H), 6.96 (m, 1H), 7.07-7.14 (m, 4H), 7.23 (m, 2H), 7.61 (d, 1H, J = 8.4 Hz), 7.80 (d, 1H, J = 9.0 Hz), 8.45 (s, 1H), 8.47 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.2, 14.3, 28.4, 28.6, 28.9, 30.9, 40.9, 41.0, 45.0, 45.1, 51.9, 52.0, 52.9, 53.5, 57.6, 61.6, 61.7, 112.0, 112.3, 113.3, 114.9, 115.0, 115.4, 115.5, 122.2, 122.4, 122.9, 123.5, 123.7, 123.8, 124.1, 124.2, 124.4, 124.5, 125.5, 126.0, 127.2, 128.9, 129.0, 129.1, 129.2, 130.4, 131.6, 131.7, 136.1, 159.7, 159.8, 160.9, 161.4, 163.2, 164.1, 164.7, 168.0, 168.2.

Ethyl 3-(2-(*tert*-butylamino)-1-(*N*-(3,4-difluorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (17e): yellow solid (79 mg, yield: 78%). HPLC/MS: t_R = 11.48 min; m/z = 506.0 [M+H]$^+$. HRMS: $C_{25}H_{26}N_3O_4F_2ClNa$, 528.1478 (calcd.), 528.1483 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.28 (s, 9H), 1.30 (s, 6H), 1.37-1.39 (m, 6H), 4.17 (ABd, 1H, J = 15.6 Hz), 4.30-4.36 (m, 4H), 4.58 (ABd, 1H, J = 16.2 Hz), 4.98 (ABd, 1H, J = 15.6 Hz), 5.62 (m, 2H), 6.15 (m, 2H), 6.39 (m, 1H), 6.47 (m, 1H), 6.65-6.78 (m, 4H), 7.15-7.17 (m, 2H), 7.35 (m, 2H), 7.68 (d, 1H, J = 9.0 Hz), 7.86 (d, 1H, J = 9.0 Hz), 8.41 (s, 1H), 8.45 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.3, 14.4, 28.5, 28.6, 28.9, 30.8, 45.8, 49.2, 52.0, 52.2, 52.4, 57.0, 61.7, 61.8, 112.1, 112.4, 113.1, 114.5, 114.6, 114.8, 116.1, 116.2, 116.3,

116.4, 116.5, 121.6, 122.1, 122.4, 122.5, 122.7, 123.2, 124.6, 125.4, 126.3, 127.2, 131.8, 131.9, 134.4, 134.5, 134.8, 136.0, 148.2, 148.3, 148.8, 148.9, 150.4, 150.5, 160.6, 160.7, 163.5, 164.6, 168.1.

Ethyl 3-(2-(*tert*-butylamino)-1-(*N*-(2,4-difluorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (17f): yellow solid (68 mg, yield: 67%). HRMS: $C_{25}H_{26}ClF_2N_3O_4Na$, 528.1478 (calcd.), 528.1456 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.26 (s, 9H), 1.32 (s, 6H), 1.36-1.42 (m, 6H), 4.36-4.50 (m, 7H), 4.60 (ABd, 1H, J = 16.2 Hz), 4.98 (ABd, 1H, J = 16.2 Hz), 5.55 (m, 2H), 6.13 (s, 1H), 6.36 (m, 1H), 6.43 (m, 1H), 6.50 (m, 1H), 6.59 (m, 1H), 6.66-6.69 (m, 2H), 6.82-6.86 (m, 2H), 7.11-7.19 (m, 3H), 7.33-7.36 (m, 2H), 7.66 (d, 1H, J = 9.0 Hz), 7.85 (d, 1H, J = 9.0 Hz), 8.24 (s, 1H), 8.44 (m, 2H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.3, 14.4, 28.5, 28.6, 28.9, 30.9, 35.6, 40.3, 44.5, 51.9, 52.1, 52.7, 57.3, 61.7, 61.8, 103.1, 103.8, 103.9, 110.3, 110.5, 111.1, 111.5, 111.9, 122.1, 113.3, 115.4, 122.4, 122.7, 122.9, 124.5, 125.5, 126.0, 127.2, 131.2, 131.8, 132.0, 136.0, 160.7, 160.8, 161.2, 163.0, 163.9, 164.5, 167.8, 168.1.

Ethyl 3-(2-(*tert*-butylamino)-1-(*N*-(2,3-difluorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (17g): yellow solid (66 mg, yield: 65%). HRMS: $C_{25}H_{26}ClF_2N_3O_4Na$, 528.1478 (calcd.), 528.1489 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.27 (s, 9H), 1.32 (s, 6H), 1.35-1.40 (m, 6H), 4.34-4.39 (m, 4H), 4.50-4.57 (m, 2H), 4.65 (ABd, 1H, J = 16.2 Hz), 5.03 (ABd, 1H, J = 15.6 Hz), 5.57 (s, 1H), 5.62 (s, 1H), 6.15 (m, 2H), 6.63 (m, 1H), 6.67 (s, 1H), 6.86 (m, 2H), 6.95 (m, 2H), 7.11-7.16 (m, 2H), 7.31-7.33 (m, 2H), 7.66 (d, 1H, J = 8.4 Hz), 7.85 (d, 1H, J = 9.0 Hz), 8.45 (m, 2H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.2, 14.3, 28.4, 28.6, 40.6, 44.7, 52.0, 52.1, 52.7, 57.4, 61.7, 61.9, 111.9, 112.1, 113.2, 115.3, 115.9, 116.0, 116.1, 116.2, 122.3, 122.5, 122.7, 122.8, 123.2, 123.3, 123.8, 124.5,

124.9, 125.5, 126.0, 126.4, 126.9, 127.0, 127.2, 131.9, 132.1, 135.9, 147.6, 149.3, 150.9, 160.8, 163.9, 164.5, 167.7, 168.0.

Ethyl 3-(2-(tert-butylamino)-1-(*N*-(2,5-difluorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (17h): yellow solid (46 mg, yield: 46%). HPLC/MS: t_R = 10.62 min; m/z = 406.3 [M+H]$^+$. HRMS: $C_{25}H_{26}ClF_2N_3O_4Na$, 528.1478 (calcd.), 528.1469 (found). ^{1}H NMR (400 MHz, CD$_2$Cl$_2$, a mixture of rotamers): 1.30 (s, 9H), 1.32 (s, 5H), 1.41-1.47 (m, 4H), 4.38-4.59 (m, 6H), 4.62 (ABd, 1H, J = 16.8 Hz), 4.95 (ABd, 1H, J = 16.0 Hz), 5.48 (s, 1H), 6.19 (s, 1H), 6.28 (m, 1H), 6.66 (s, 1H), 6.69-6.79 (m, 4H), 7.00-7.12 (m, 4H), 7.17-7.21 (m, 2H), 7.39 (m, 2H), 7.73 (d, 1H, J = 8.8 Hz), 7.88 (d, 1H, J = 8.8 Hz), 8.29 (m, 2H), 8.41 (s, 1H), 8.48 (s, 1H), 9.03 (s, 1H), 9.19 (s, 1H). ^{13}C NMR (100 MHz, CD$_2$Cl$_2$, a mixture of rotamers): 14.0, 14.1, 28.2, 28.3, 35.6, 40.2, 40.3, 44.2, 51.2, 51.9, 52.5, 57.0, 61.8, 61.9, 111.6, 111.8, 113.4, 114.5, 114.7, 115.3, 115.4, 115.5, 115.8, 115.9, 116.0, 116.2, 116.3, 116.5, 122.4, 122.5, 122.6, 123.0, 124.7, 125.6, 126.4, 127.3, 131.7, 131.9, 135.7, 160.6, 160.9, 163.6, 164.2, 167.7.

Ethyl 3-(2-(*tert*-butylamino)-1-(*N*-(3,5-difluorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (17i): yellow solid (75 mg, yield: 74%). HRMS: $C_{25}H_{26}ClF_2N_3O_4Na$, 528.1478 (calcd.), 528.1453 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.31 (s, 9H), 1.33 (s, 6H), 1.41-1.44 (m, 6H), 4.21 (ABd, 1H, J = 15.6 Hz), 4.34-4.50 (m, 6H), 5.02 (ABd, 1H, J = 16.2 Hz), 5.49 (m, 2H), 6.06 (m, 2H), 6.17 (s, 1H), 6.33 (m, 2H), 6.50 (m, 2H), 6.74 (s, 1H), 6.83 (m, 1H), 7.19-7.21 (m, 2H), 7.36-7.37 (m, 2H), 7.70 (d, 1H, J = 9.0 Hz), 7.90 (d, 1H, J = 8.4 Hz), 8.42 (s, 1H), 8.49 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.4, 28.5, 28.6, 28.9, 30.9, 41.3, 46.0, 49.4, 50.9, 52.0, 52.2, 52.3, 56.9, 61.9, 62.0, 102.1, 102.3, 108.3, 108.5, 109.8, 110.0, 111.9, 112.2, 113.1, 115.1, 122.2, 122.5, 122.8, 122.9, 124.7, 125.5, 126.4, 127.3, 132.1, 132.3, 135.8, 141.5, 160.5, 161.2, 163.4, 167.9, 168.0.

Ethyl 3-(2-(*tert*-butylamino)-1-(*N*-(2,6-difluorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (17j): yellow solid (75 mg, yield: 74%). HRMS: $C_{25}H_{26}ClF_2N_3O_4Na$, 528.1478 (calcd.), 528.1467 (found). ^1H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.21 (s, 9H), 1.33 (s, 9H), 1.36 (m, 6H), 4.25-4.31 (m, 4H), 4.49-4.57 (m, 2H), 4.68 (ABd, 1H, J = 15.0 Hz), 5.13 (ABd, 1H, J = 15.0 Hz), 5.65 (s, 1H), 5.81 (s, 1H), 6.02 (s, 1H), 6.55-6.58 (m, 3H), 6.80 (m, 2H), 6.89 (m, 1H), 7.05 (m, 3H), 7.20 (m, 1H), 7.63 (d, 1H, J = 9.0 Hz), 7.70 (d, 1H, J = 9.0 Hz), 8.29 (s, 1H), 8.44 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.2, 14.3, 28.4, 28.6, 35.4, 39.0, 51.9, 53.7, 57.2, 61.5, 61.7, 111.0, 111.1, 111.2, 111.3, 111.6, 111.9, 112.3, 112.8, 113.0, 115.1, 122.0, 122.2, 122.4, 122.8, 124.7, 125.6, 125.9, 127.2, 129.6, 131.4, 131.6, 136.1, 136.2, 160.9, 161.0, 164.0, 164.1, 167.8, 168.2.

Ethyl 3-(2-(*tert*-butylamino)-2-oxo-1-(*N*-(2,3,4-trifluorobenzyl)formamido)ethyl)-6-chloro-1*H*-indole-2-carboxylate (17k): yellow solid (40 mg, yield: 38%). HRMS: $C_{25}H_{25}ClF_3N_3O_4Na$, 546.1383 (calcd.), 546.1398 (found). ^1H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.28 (s, 9H), 1.33 (s, 6H), 1.40-1.46 (m, 6H), 4.44-4.54 (m, 6H), 4.65 (ABd, 1H, J = 16.2 Hz), 5.00 (ABd, 1H, J = 15.6 Hz), 5.46 (s, 1H), 6.13-6.16 (m, 2H), 6.51 (m, 1H), 6.66 (s, 1H), 6.75-6.79 (m, 1H), 6.94-6.99 (m, 2H), 7.11-7.19 (m, 2H), 7.38 (s, 1H), 7.69 (d, 1H, J = 8.4 Hz), 7.88 (d, 1H, J = 9.0 Hz), 8.06 (s, 1H), 8.29 (m, 2H), 8.44 (s, 1H), 9.35 (br.s, 1H), 9.44 (br.s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.3, 14.4, 28.5, 28.6, 28.9, 30.9, 35.6, 40.3, 44.4, 52.0, 52.2, 52.5, 57.2, 61.8, 62.0, 111.0, 111.2, 111.6, 111.7, 111.8, 112.0, 112.2, 112.3, 113.3, 115.5, 122.4, 122.7, 122.9, 123.0, 123.7, 124.5, 125.4, 125.9, 127.1, 132.1, 132.4, 135.8, 160.6, 160.7, 161.1, 162.9, 163.7, 164.4, 167.5, 167.8.

Ethyl 3-(2-(*tert*-butylamino)-2-oxo-1-(*N*-(2,4,5-trifluorobenzyl)formamido)ethyl)-6-chloro-1*H*-indole-2-carboxylate (17l): yellow solid (42 mg, yield: 40%). HRMS: $C_{25}H_{25}ClF_3N_3O_4Na$,

546.1383 (calcd.), 546.1370 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.29 (s, 9H), 1.31 (s, 5H), 3.36-4.47 (m, 6H), 4.58 (ABd, 1H, J = 16.2 Hz), 4.92 (ABd, 1H, J = 15.6 Hz), 5.58 (m, 2H), 6.18 (s, 1H), 6.27 (m, 1H), 6.56-6.64 (m, 2H), 6.70 (s, 1H), 6.88-6.94 (m, 2H), 7.12-7.23 (m, 3H), 7.35 (s, 1H), 7.38 (s, 1H), 7.67 (d, 1H, J = 8.4 Hz), 7.85 (d, 1H, J = 9.0 Hz), 8.27 (m, 1H), 8.41 (s, 1H), 8.44 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.2, 14.3, 28.5, 28.6, 28.9, 30.8, 35.3, 40.1, 44.0, 52.0, 52.2, 52.5, 57.1, 61.8, 61.9, 104.5, 104.6, 104.8, 105.5, 105.6, 112.1, 112.2, 113.1, 115.0, 122.2, 122.6, 122.7, 124.4, 125.3, 126.1, 127.2, 132.1, 136.0, 160.8, 161.5, 163.3, 163.8, 164.6, 167.8, 168.0.

Ethyl 3-(2-(*tert*-butylamino)-2-oxo-1-(*N*-(3,4,5-trifluorobenzyl)formamido)ethyl)-6-chloro-1*H*-indole-2-carboxylate (17m): yellow solid (80 mg, yield: 76%). HRMS: C$_{25}$H$_{25}$ClF$_3$N$_3$O$_4$Na, 546.1383 (calcd.), 546.1384 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.32 (s, 14H), 1.41-1.44 (m, 5H), 4.14 (ABd, 1H, J = 15.6 Hz), 4.33 (ABd, 1H, J = 16.8 Hz), 4.39-4.43 (m, 3H), 4.58 (ABd, 1H, J = 16.2 Hz), 5.01 (ABd, 1H, J = 15.6 Hz), 6.17 (m, 2H), 6.39-6.42 (m, 2H), 6.75 (s, 1H), 7.19-7.21 (m, 2H), 7.38-7.40 (m, 2H), 7.71 (d, 1H, J = 8.4 Hz), 7.89 (d, 1H, J = 8.4 Hz), 8.40 (s, 1H), 8.46 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.3, 28.5, 28.6, 45.7, 49.1, 52.1, 52.2, 56.8, 61.9, 62.0, 109.5, 109.6, 109.7, 109.9, 110.9, 111.0, 111.1, 112.0, 112.3, 113.2, 115.0, 122.1, 122.4, 122.8, 123.0, 124.6, 125.4, 126.3, 127.2, 132.1, 132.3, 135.9, 160.5, 163.4, 164.4, 167.9.

Ethyl 3-(2-(*tert*-butylamino)-2-oxo-1-(*N*-(2,3,6-trifluorobenzyl)formamido)ethyl)-6-chloro-1*H*-indole-2-carboxylate (17n): yellow solid (72 mg, yield: 69%). HRMS: C$_{25}$H$_{25}$ClF$_3$N$_3$O$_4$Na, 546.1383 (calcd.), 546.1387 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.24 (s, 9H), 1.33 (s, 7H), 1.37-1.40 (m, 6H), 4.31-4.37 (m, 4H), 4.61 (m, 2H), 4.70 (ABd, 1H, J = 15.0 Hz), 5.11 (ABd, 1H, J = 15.0 Hz), 5.56 (s, 1H), 5.63 (s, 1H), 6.05 (s, 1H), 6.48 (m, 1H),

6.57 (s, 1H), 6.72 (m, 1H), 6.83-6.88 (m, 2H), 6.98-7.02 (m, 1H), 7.09-7.10 (m, 2H), 7.34 (m, 2H), 7.67 (d, 1H, J = 8.4 Hz), 7.76 (d, 1H, J = 9.0 Hz), 8.29 (s, 1H), 8.47 (s, 1H).^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.1, 14.3, 28.4, 28.6, 28.9, 30.9, 35.5, 39.4, 50.7, 51.9, 52.0, 53.3, 57.0, 61.7, 61.9, 111.8, 112.1, 112.7, 115.1, 122.3, 122.4, 122.7, 122.8, 124.7, 125.5, 126.0, 127.2, 131.8, 131.9, 136.0, 136.1, 160.7, 160.8, 160.9, 161.0, 163.1, 163.8, 163.9, 167.6, 168.0.

Ethyl 3-(2-(*tert*-butylamino)-2-oxo-1-(*N*-(2,4,6-trifluorobenzyl)formamido)ethyl)-6-chloro-1*H*-indole-2-carboxylate (17o): yellow solid (64 mg, yield: 61%). HRMS: C$_{25}$H$_{25}$ClF$_3$N$_3$O$_4$K, 562.1123 (calcd.), 562.1135 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.24 (s, 9H), 1.34 (s, 9H), 1.36-1.43 (m, 6H), 4.34-4.39 (m, 4H), 4.53-4.55 (m, 2H), 4.64 (ABd, 1H, J = 14.4 Hz), 5.08 (ABd, 1H, J = 14.4 Hz), 5.48 (s, 1H), 5.59 (s, 1H), 6.00 (s, 1H), 6.33-6.36 (m, 2H), 6.56-6.60 (m, 3H), 7.10-7.12 (m, 2H), 7.34 (m, 2H), 7.68 (d, 1H, J = 8.4 Hz), 7.78 (d, 1H, J = 9.0 Hz), 8.28 (s, 1H), 8.45 (s, 1H), 9.46 (s, 1H), 9.68 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.2, 14.4, 28.5, 28.6, 35.0, 38.8, 52.0, 53.4, 56.9, 61.7, 61.9, 99.9, 100.0, 100.1, 111.7, 112.1, 113.1, 115.4, 122.3, 122.5, 122.8, 123.0, 124.7, 125.6, 125.8, 127.1, 131.9, 132.1, 135.9, 160.7, 160.9, 163.8, 167.6, 168.0.

Ethyl 3-(2-(*tert*-butylamino)-2-oxo-1-(*N*-(2,3,5-trifluorobenzyl)formamido)ethyl)-6-chloro-1*H*-indole-2-carboxylate (17p): yellow solid (50 mg, yield: 48%). HRMS: C$_{25}$H$_{25}$ClF$_3$N$_3$O$_4$Na, 546.1383 (calcd.), 546.1355 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.30 (s, 9H), 1.33 (s, 5H), 4.43-4.57 (m, 6H), 4.66 (ABd, 1H, J = 16.8 Hz), 5.01 (ABd, 1H, J = 16.2 Hz), 5.46 (m, 2H), 5.99 (m, 1H), 6.19 (s, 1H), 6.22 (m, 1H), 6.59-6.66 (m, 3H), 6.70 (s, 1H), 6.87-6.91 (m, 2H), 7.15-7.20 (m, 2H), 7.36-7.38 (m, 2H), 7.70 (d, 1H, J = 9.0 Hz), 7.88 (d, 1H, J = 9.0 Hz), 8.06 (s, 1H), 8.30 (m, 1H), 8.43 (s, 1H), 8.48 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a

mixture of rotamers): 14.3, 14.4, 28.5, 28.6, 28.9, 30.9, 35.6, 40.3, 44.3, 52.0, 52.2, 52.4, 57.1, 61.9, 62.1, 104.7, 104.8, 104.9, 105.0, 110.5, 110.7, 111.1, 111.3, 111.9, 112.0, 113.0, 115.2, 122.3, 122.6, 122.8, 123.0, 124.4, 125.4, 126.1, 127.2, 132.2, 132.4, 135.7, 135.8, 160.6, 160.7, 161.2, 162.9, 163.7, 164.4, 167.6, 167.8.

Ethyl 3-(2-(*tert*-butylamino)-2-oxo-1-(*N*-(2,3,5,6-tetrafluorobenzyl)formamido)ethyl)-6-chloro-1*H*-indole-2-carboxylate (17q): yellow solid (79 mg, yield: 73%). HPLC/MS: t_R = 11.03 min; m/z = 541.9 [M+H]$^+$. HRMS: $C_{25}H_{24}ClF_4N_3O_4Na$, 564.1289 (calcd.), 564.1276 (found). ^{1}H NMR (400 MHz, CDCl$_3$, a mixture of rotamers): 1.24 (s, 9H), 1.30 (s, 5H), 1.32-1.38 (m, 6H), 4.24-4.35 (m, 3H), 4.58-4.63 (m, 2H), 4.74 (ABd, 1H, J = 15.2 Hz), 5.07 (ABd, 1H, J = 15.2 Hz), 5.70 (s, 1H), 5.72 (s, 1H), 6.08 (s, 1H), 6.58 (s, 1H), 6.75 (m, 1H), 6.88 (m, 1H), 7.00 (m, 1H), 7.06-7.09 (m, 2H), 7.35 (m, 2H), 7.65 (d, 1H, J = 8.8 Hz), 7.72 (d, 1H, J = 8.8 Hz), 8.15 (s, 1H), 8.25 (s, 1H), 8.46 (s, 1H). ^{13}C NMR (100 MHz, CDCl$_3$, a mixture of rotamers): 14.0, 14.2, 28.4, 28.5, 28.8, 30.8, 35.6, 50.5, 51.4, 51.9, 52.1, 53.2, 56.9, 61.6, 61.8, 104.9, 105.1, 105.4, 105.6, 112.0, 112.3, 114.6, 116.0, 122.1, 122.3, 122.4, 1, 125.3, 126.1, 127.2, 131.8, 131.9, 136.1, 136.2, 160.8, 160.9, 161.2, 163.2, 164.0, 167.7, 168.0.

Ethyl 3-(2-(*tert*-butylamino)-2-oxo-1-(*N*-(2,3,4,6-tetrafluorobenzyl)formamido)ethyl)-6-chloro-1*H*-indole-2-carboxylate (17r): yellow solid (60 mg, yield: 55%). HRMS: $C_{25}H_{24}ClF_4N_3O_4Na$, 564.1289 (calcd.), 564.1314 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.25 (s, 9H), 1.33 (s, 6H), 1.35-1.40 (m, 6H), 4.32-4.37 (m, 4H), 4.59 (m, 2H), 4.69 (ABd, 1H, J = 14.4 Hz), 5.04 (ABd, 1H, J = 15.0 Hz), 5.53 (s, 1H), 5.62 (s, 1H), 6.04 (s, 1H), 6.43 (m, 1H), 6.57 (s, 1H), 6.66 (m, 1H), 7.10-7.12 (m, 2H), 7.38 (m, 2H), 7.68 (d, 1H, J = 8.4 Hz), 7.77 (d, 1H, J = 8.4 Hz), 8.25 (s, 1H), 8.46 (s, 1H), 9.82 (s, 1H), 10.04 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.2, 14.3, 28.4, 28.6, 30.8, 35.3, 39.1, 45.6, 52.0,

52.1, 53.2, 56.8, 61.7, 61.9, 100.3, 100.5, 111.8, 112.2, 112.8, 115.0, 122.3, 122.4, 122.7, 122.8, 124.6, 125.4, 125.9, 127.0, 132.0, 132.1, 135.9, 136.0, 160.7, 163.9, 167.6, 167.9.

Ethyl 3-(2-(*tert*-butylamino)-2-oxo-1-(*N*-(2,3,4,5-tetrafluorobenzyl)formamido)ethyl)-6-chloro-1*H*-indole-2-carboxylate (17s): yellow solid (44 mg, yield: 41%). HRMS: $C_{25}H_{24}ClF_4N_3O_4Na$, 564.1289 (calcd.), 564.1298 (found). ^1H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.22 (s, 9H), 1.31 (s, 7H), 1.38-1.41 (m, 6H), 4.34-4.39 (m, 4H), 4.48-4.51 (m, 2H), 4.62 (ABd, 1H, J = 16.2 Hz), 4.83 (ABd, 1H, J = 15.0 Hz), 5.50 (s, 1H), 5.57 (s, 1H), 6.14 (s, 1H), 6.56 (m, 2H), 6.73 (s, 1H), 6.95-6.98 (m, 2H), 7.01-7.05 (m, 3H), 7.15-7.17 (m, 5H), 7.30-7.32 (m, 2H), 7.62 (d, 1H, J = 9.0 Hz), 7.88 (d, 1H, J = 8.4 Hz), 8.05 (s, 1H), 8.29 (m, 1H), 8.45 (s, 1H), 8.53 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.4, 28.4, 28.6, 28.9, 30.9, 46.7, 50.1, 51.9, 52.6, 57.6, 61.6, 61.7, 111.8, 112.2, 113.5, 115.6, 122.3, 122.5, 122.9, 124.9, 125.7, 125.8, 126.3, 127.1, 127.3, 127.8, 127.9, 128.4, 128.8, 131.7, 131.9, 135.9, 136.0, 137.4, 137.5, 160.6, 160.7, 161.4, 163.0, 163.6, 164.7, 168.1, 168.2.

Ethyl 3-(2-(*tert*-butylamino)-2-oxo-1-(*N*-(perfluorobenzyl)formamido)ethyl)-6-chloro-1*H*-indole-2-carboxylate (17t): yellowish solid (68 mg, yield: 61%). HPLC/MS: t_R = 11.76 min; m/z = 559.7 [M+H]$^+$. HRMS: $C_{25}H_{23}ClF_5N_3O_4Na$, 582.1195 (calcd.), 582.1151 (found). ^1H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.28 (s, 9H), 1.33 (s, 5H), 1.38-1.43 (m, 5H), 4.36-4.43 (m, 3H), 4.65 (m, 1H), 4.76 (ABd, 1H, J = 15.0 Hz), 5.03 (ABd, 1H, J = 15.0 Hz), 5.44 (s, 1H), 5.51 (s, 1H), 6.05 (s, 1H), 6.57 (s, 1H), 7.14 (m, 1H), 7.38 (m, 1H), 7.69 (d, 1H, J = 9.0 Hz), 7.78 (d, 1H, J = 8.4 Hz), 8.25 (s, 1H), 8.47 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.2, 14.3, 28.5, 28.6, 35.2, 39.1, 50.8, 52.1, 52.2, 53.0, 56.8, 61.8, 62.1, 111.8, 112.1, 112.8, 115.0, 122.3, 122.7, 123.0, 124.6, 125.3, 125.9, 127.0, 132.3, 132.4, 135.8, 135.9, 160.6, 160.7, 160.9, 163.7, 167.5, 167.7.

General procedure for the synthesis of acid compounds: The ester compound **17** was treated

with LiOH in EtOH/water (1:1), and stirring under RT for 2 days. Then the reaction mixture was

acidified with 1M HCl (pH ~ 6). The mixture was extracted with dichloromethane (10 mL x 3).

The combined organic layer was dried over sodium sulfate, and filtered. After evaporation of the

solvent, compound **18** was obtained without further purification.

3-(1-(*N*-Benzylformamido)-2-(*tert*-butylamino)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylic

acid (18a): yellowish solid (14 mg, 83%). HPLC/MS: t_R = 11.37 min; m/z = 441.6 [M+H]$^+$.

HRMS: $C_{23}H_{24}ClN_3O_4Na$, 464.1353 (calcd.), 464.1334 (found). ^{1}H NMR (400 MHz, CD$_3$OD, a

mixture of rotamers): 1.21 (s, 9H), 1.31 (s, 6H), 4.30-4.36 (m, 2H), 4.71 (ABd, 1H, J = 16.4 Hz),

5.13 (ABd, 1H, J = 15.2 Hz), 6.29 (s, 1H), 6.53 (m, 1H), 6.81 (s, 1H), 6.92-7.00 (m, 4H), 7.06-

7.13 (m, 4H), 7.36 (m, 1H), 7.40 (m, 1H), 7.78 (d, 1H, J = 8.8 Hz), 7.84 (d, 1H, J = 8.8 Hz),

8.39 (s, 1H), 8.44 (s, 1H). ^{13}C NMR (100 MHz, CD$_3$OD, a mixture of rotamers): 27.4, 27.5, 50.0,

51.0, 51.1, 53.1, 56.9, 111.7, 111.8, 120.8, 120.9, 121.8, 122.0, 124.8, 125.3, 126.5, 127.3, 127.5,

130.2, 136.2, 136.7, 137.5, 169.6.

3-(2-(*tert*-Butylamino)-1-(*N*-(4-fluorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-

carboxylic acid (18b): white solid (30 mg, 62%). HPLC/MS: t_R = 11.45 min; m/z = 459.8

[M+H]$^+$. HRMS: $C_{23}H_{23}ClFN_3O_4Na$, 482.1259 (calcd.), 482.1286 (found). ^{1}H NMR (400 MHz,

CD$_3$OD, a mixture of rotamers):1.24 (s, 9H), 1.31 (s, 5H), 4.27-4.32 (m, 2H), 4.68 (ABd, 1H, J

= 16.0 Hz), 5.12 (ABd, 1H, J = 15.2 Hz), 6.25 (s, 1H), 6.48-6.51 (m, 1H), 6.62-6.67 (m, 1H),

6.74-6.79 (m, 3H), 6.86-6.89 (m, 2H), 7.10-7.14 (m, 2H), 7.39-7.42 (m, 2H), 7.56 (s, 1H), 7.80

(d, 1H, J = 8.8 Hz), 7.84 (d, 1H, J = 8.8 Hz), 8.36 (s, 1H), 8.46 (s, 1H). ^{13}C NMR (100 MHz,

CD$_3$OD, a mixture of rotamers): 27.4, 27.5, 46.1, 49.4, 51.1, 51.2, 52.7, 56.8, 56.9, 111.8, 111.9,

113.7, 113.9, 114.0, 114.1, 120.9, 121.1, 121.8, 122.0, 124.7, 125.5, 127.0, 127.1, 129.0, 129.1, 130.3, 130.4, 132.9, 133.6, 136.3, 160.6, 163.0, 164.6, 165.2, 169.6, 169.8.

3-(2-(*tert*-Butylamino)-1-(*N*-(3-fluorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylic acid (18c): yellowish solid (65 mg, 91%). HPLC/MS: t_R = 11.30 min; m/z = 459.9 [M+H]$^+$. HRMS: $C_{23}H_{23}ClFN_3O_4Na$, 482.1259 (calcd.), 482.1257 (found). ^{1}H NMR (600 MHz, CD$_3$OD, a mixture of rotamers): 1.13 (s, 9H), 1.19 (s, 5H), 4.17-4.19 (m, 2H), 4.60 (ABd, 1H, J = 16.8 Hz), 5.03 (ABd, 1H, J = 15.6 Hz), 6.17 (m, 2H), 6.46-6.50 (m, 2H), 6.58 (m, 1H), 6.65-6.68 (m, 2H), 6.79 (m, 1H), 6.88-6.90 (m, 1H), 6.98-7.01 (m, 2H), 7.26-7.29 (m, 2H), 7.41 (s, 1H), 7.47 (s, 1H), 7.68 (m, 1H), 7.73 (m, 1H), 8.28 (s, 1H), 8.34 (s, 1H). ^{13}C NMR (150 MHz, CD$_3$OD, a mixture of rotamers): 28.9, 29.0, 47.8, 49.5, 51.0, 52.7, 54.1, 58.4, 113.2, 113.3, 113.5, 113.8, 114.4, 114.5, 114.6, 115.0, 115.2, 115.3, 122.4, 122.6, 123.1, 123.3, 124.1, 126.1, 127.0, 129.6, 130.4, 131.7, 131.8, 137.7, 141.2, 142.0, 163.0, 164.6, 166.1, 166.8, 171.1, 171.3.

3-(2-(*tert*-Butylamino)-1-(*N*-(2-fluorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylic acid (18d): yellowish solid (55 mg, 78%). HPLC/MS: t_R = 11.27 min; m/z = 459.8 [M+H]$^+$. HRMS: $C_{23}H_{23}ClFN_3O_4Na$, 482.1259 (calcd.), 482.1237 (found). ^{1}H NMR (600 MHz, CD$_3$OD, a mixture of rotamers): 1.10 (s, 9H), 1.18 (s, 5H), 4.39 (ABd, 1H, J = 16.2 Hz), 4.52 (ABd, 1H, J = 15.6 Hz), 4.61 (ABd, 1H, J = 16.8 Hz), 4.92 (ABd, 1H, J = 15.6 Hz), 6.16 (s, 1H), 6.35 (m, 1H), 6.56-6.62 (m, 2H), 6.68 (m, 1H), 6.79 (m, 1H), 6.90-6.99 (m, 4H), 7.26 (m, 2H), 7.35 (s, 1H), 7.49 (s, 1H), 7.64 (m, 1H), 7.68 (m, 1H), 8.24 (s, 1H), 8.34 (s, 1H). ^{13}C NMR (150 MHz, CD$_3$OD, a mixture of rotamers): 28.8, 28.9, 41.9, 42.0, 45.8, 45.9, 52.7, 54.3, 58.5, 113.1, 113.2, 115.5, 115.6, 115.8, 122.3, 122.5, 123.2, 123.5, 124.4, 124.5, 124.7, 124.8, 124.9, 126.0, 126.9, 129.1, 129.4, 129.8, 129.9, 130.9, 131.8, 131.9, 137.7, 161.0, 162.6, 163.6, 163.9, 166.6, 166.7, 170.8, 171.2.

3-(2-(*tert*-Butylamino)-1-(*N*-(3,4-difluorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-

indole-2-carboxylic acid (18e): yellowish solid (62 mg, 91%). HPLC/MS: t_R = 10.85 min; *m/z* =

477.9 [M+H]$^+$. HRMS: $C_{22}H_{22}N_3O_4F_2ClNa$, 500.1165 (calcd.), 500.1166 (found). ^{1}H NMR (400

MHz, CD$_3$OD, a mixture of rotamers): 1.20 (s, 9H), 1.26 (s, 3H), 4.32-4.40 (m, 2H), 4.59 (ABd,

1H, *J* = 16.4 Hz), 5.01 (ABd, 1H, *J* = 15.2 Hz), 6.39-6.44 (m, 1H), 6.58 (s, 1H), 6.76-6.78 (m,

2H), 6.85-6.97 (m, 2H), 7.00-7.02 (m, 2H), 7.09 (s, 1H), 7.32-7.37 (m, 2H), 7.69 (d, 1H, *J* = 8.8

Hz), 7.73 (d, 1H, *J* = 8.8 Hz), 8.34 (s, 1H), 8.37 (s, 1H). ^{13}C NMR (100 MHz, CD$_3$OD, a mixture

of rotamers): 27.4, 27.5, 46.3, 49.0, 50.9, 53.1, 57.1, 108.2, 109.8, 111.4, 111.5, 116.0, 116.2,

116.3, 116.5, 120.1, 120.3, 121.2, 121.5, 123.8, 123.9, 125.0, 128.7, 128.8, 134.4, 134.5, 135.3,

147.9, 148.3, 148.4, 150.3, 150.4, 150.8, 164.9, 165.4, 167.6, 170.0, 170.4.

3-(2-(*tert*-Butylamino)-1-(*N*-(2,4-difluorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-

indole-2-carboxylic acid (18f): yellowish solid (50 mg, 78%). HPLC/MS: t_R = 11.64 min; *m/z* =

477.5 [M+H]$^+$. HRMS: $C_{23}H_{22}ClF_2N_3O_4Na$, 500.1165 (calcd.), 500.1190 (found). ^{1}H NMR (400

MHz, CD$_3$OD, a mixture of rotamers): 1.21 (s, 9H), 1.27 (s, 5H), 4.44 (ABd, 1H, *J* = 16.4 Hz),

4.57 (ABd, 1H, *J* = 15.2 Hz), 4.66 (ABd, 1H, *J* = 16.4 Hz), 4.95 (ABd, 1H, *J* = 15.6 Hz), 6.26

(s, 1H), 6.43-6.52 (m, 2H), 6.60-6.70 (m, 3H), 7.02-7.09 (m, 3H), 7.37 (m, 2H), 7.73 (d, 1H, *J* =

8.8 Hz), 7.79 (d, 1H, *J* = 8.8 Hz), 8.31 (s, 1H), 8.42 (s, 1H). ^{13}C NMR (100 MHz, CD$_3$OD, a

mixture of rotamers): 27.4, 27.5, 40.0, 44.0, 51.1, 51.2, 52.9, 57.0, 102.2, 102.5, 102.7, 110.0,

110.1, 110.4, 111.7, 111.8, 113.7, 119.7, 119.8, 120.9, 121.1, 121.7, 122.0, 124.6, 125.4, 129.1,

130.4, 130.6, 130.7, 136.2, 136.3, 159.1, 159.2, 163.2, 163.3, 165.2, 169.4, 169.7.

3-(2-(*tert*-Butylamino)-1-(*N*-(2,3-difluorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-

indole-2-carboxylic acid (18g): white solid (32 mg, 55%). HPLC/MS: t_R = 12.13 min; *m/z* =

477.4 [M+H]$^+$. HRMS: $C_{23}H_{23}ClF_2N_3O_4$, 478.1345 (calcd.), 478.1375 (found). ^{1}H NMR (400

MHz, CD$_3$OD, a mixture of rotamers): 1.25 (s, 9H), 1.31 (s, 5H), 4.54 (ABd, 1H, J = 16.8 Hz), 4.65 (ABd, 1H, J = 15.6 Hz), 4.76 (ABd, 1H, J = 16.8 Hz), 5.06 (ABd, 1H, J = 15.6 Hz), 6.25-6.29 (m, 2H), 6.69-6.74 (m, 2H), 6.84-6.91 (m, 2H), 6.94-7.01 (m, 1H), 7.07-7.11 (m, 2H), 7.40 (m, 2H), 7.77 (d, 1H, J = 8.8 Hz), 7.82 (d, 1H, J = 8.8 Hz), 8.36 (s, 1H), 8.48 (s, 1H). ^{13}C NMR (100 MHz, CD$_3$OD, a mixture of rotamers): 27.4, 27.5, 40.1, 44.1, 51.1, 51.3, 52.8, 57.0, 111.7, 111.8, 112.3, 114.0, 115.2, 115.4, 121.0, 121.2, 121.7, 121.9, 122.8, 123.0, 123.3, 123.4, 124.5, 125.4, 126.3, 126.4, 127.3, 127.4, 130.4, 130.5, 136.2, 136.3, 165.0, 165.2, 169.3, 169.6.

3-(2-(*tert*-Butylamino)-1-(*N*-(2,5-difluorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylic acid (18h): yellowish solid (30 mg, 74%). HPLC/MS: t_R = 11.73 min; m/z = 477.8 [M+H]$^+$. HRMS: C$_{23}$H$_{23}$ClF$_2$N$_3$O$_4$, 478.1345 (calcd.), 478.1321 (found). ^{1}H NMR (400 MHz, CD$_3$OD, a mixture of rotamers): 1.24 (s, 9H), 1.29 (s, 3H), 4.60-4.65 (m, 2H), 4.98 (ABd, 1H, J = 16.0 Hz), 6.42-6.46 (m, 1H), 6.68 (s, 1H), 6.71 (m, 1H), 6.80-6.90 (m, 3H), 6.97-7.01 (m, 2H), 7.06 (s, 1H), 7.34-7.37 (m, 2H), 7.69 (d, 1H, J = 8.8 Hz), 7.73 (d, 1H, J = 8.4 Hz), 8.37 (s, 1H), 8.40 (s, 1H). ^{13}C NMR (100 MHz, CD$_3$OD, a mixture of rotamers): 25.9, 26.0, 39.4, 49.4, 55.9, 108.0, 109.9, 112.65, 112.74, 113.0, 113.7, 113.96, 114.02, 114.3, 118.7, 119.6, 123.4, 127.2, 133.7, 163.9, 165.9, 168.5.

3-(2-(*tert*-Butylamino)-1-(*N*-(3,5-difluorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylic acid (18i): yellowish solid (60 mg, 93%). HPLC/MS: t_R = 11.93 min; m/z = 477.5 [M+H]$^+$. HRMS: C$_{23}$H$_{23}$ClF$_2$N$_3$O$_4$, 478.1345 (calcd.), 478.1353 (found). ^{1}H NMR (600 MHz, CD$_3$OD, a mixture of rotamers): 1.28 (s, 9H), 1.31 (s, 4H), 4.27 (ABd, 1H, J = 15.6 Hz), 4.33 (ABd, 1H, J = 16.8 Hz), 4.71 (ABd, 1H, J = 16.8 Hz), 5.14 (ABd, 1H, J = 15.6 Hz), 6.06 (m, 1H), 6.33 (s, 1H), 6.35 (m, 2H), 6.53-6.61 (m, 2H), 6.80 (s, 1H), 7.13-7.16 (m, 2H), 7.41 (m, 1H), 7.42 (m, 1H), 7.58 (s, 1H), 7.65 (s, 1H), 7.80 (d, 1H, J = 9.0 Hz), 7.84 (d, 1H, J = 8.4 Hz),

8.42 (s, 1H), 8.46 (s, 1H). ^{13}C NMR (150 MHz, CD$_3$OD, a mixture of rotamers): 27.4, 27.5, 46.1, 48.1, 49.4, 51.3, 52.6, 56.9, 101.1, 101.3, 101.4, 101.5, 108.0, 108.1, 109.4, 109.5, 109.6, 111.8, 111.9, 112.3, 113.7, 121.1, 121.2, 121.6, 121.7, 124.7, 125.5, 128.3, 128.8, 130.4, 136.2, 141.6, 141.7, 161.5, 161.6, 162.2, 162.6, 163.2, 163.3, 164.7, 165.4, 169.7.

3-(2-(*tert*-Butylamino)-1-(*N*-(2,6-difluorobenzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylic acid (18j): yellowish solid (30 mg, 64%). HPLC/MS: t_R = 10.88 min; m/z = 477.4 [M+H]$^+$. HRMS: C$_{23}$H$_{22}$ClF$_2$N$_3$O$_4$Na, 500.1165 (calcd.), 500.1121 (found). ^{1}H NMR (600 MHz, CD$_3$OD, a mixture of rotamers): 1.17 (s, 9H), 1.30 (s, 5H), 4.61 (ABd, 1H, *J* = 16.2 Hz), 4.70 (ABd, 1H, *J* = 15.6 Hz), 4.86-5.01 (m, 2H), 6.14 (s, 1H), 6.57 (m, 2H), 6.87-6.90 (m, 2H), 7.02-7.07 (m, 2H), 7.29-7.32 (m, 1H), 7.40-7.46 (m, 2H), 7.74-7.76 (m, 2H), 8.11 (s, 1H), 8.50 (s, 1H). ^{13}C NMR (150 MHz, CD$_3$OD, a mixture of rotamers): 27.4, 27.5, 34.6, 39.3, 48.5, 51.0, 51.1, 51.2, 53.6, 56.6, 110.5, 110.6, 110.7, 110.8, 110.9, 111.0, 111.5, 111.7, 111.8, 120.7, 121.1, 121.8, 122.1, 124.8, 125.7, 129.1, 129.8, 129.9, 130.3, 130.4, 136.2, 136.4, 160.3, 160.4, 161.2, 162.0, 162.8, 162.9, 164.5, 165.4, 169.0, 169.6.

3-(2-(*tert*-Butylamino)-2-oxo-1-(*N*-(2,3,4-trifluorobenzyl)formamido)ethyl)-6-chloro-1*H*-indole-2-carboxylic acid (18k): white solid (29 mg, 79%). HPLC/MS: t_R = 12.39 min; m/z = 495.6 [M+H]$^+$. HRMS: C$_{23}$H$_{21}$ClF$_3$N$_3$O$_4$Na, 518.1070 (calcd.), 518.1038 (found). ^{1}H NMR (600 MHz, CD$_3$OD, a mixture of rotamers): 1.26 (s, 9H), 1.31 (s, 5H), 4.49 (ABd, 1H, *J* = 16.2 Hz), 4.61 (ABd, 1H, *J* = 15.0 Hz), 4.73 (ABd, 1H, *J* = 16.8 Hz), 5.01 (ABd, 1H, *J* = 15.6 Hz), 6.29 (s, 1H), 6.72 (s, 1H), 6.86-6.88 (m, 2H), 7.08-7.12 (m, 2H), 7.42 (m, 2H), 7.58 (s, 1H), 7.71 (s, 1H), 7.76 (d, 1H, *J* = 8.4 Hz), 7.82 (d, 1H, *J* = 9.0 Hz), 8.33 (s, 1H), 8.48 (s, 1H). ^{13}C NMR (150 MHz, CD$_3$OD, a mixture of rotamers): 27.4, 27.5, 39.8, 43.9, 51.3, 52.0, 52.8, 57.0, 110.7, 110.8,

111.1, 111.2, 111.7, 111.8, 112.1, 113.6, 121.0, 121.2, 121.6, 121.8, 121.9, 123.6, 124.5, 125.4, 130.5, 136.1, 136.2, 165.0, 165.2, 169.4, 169.7.

3-(2-(*tert*-Butylamino)-2-oxo-1-(*N*-(2,4,5-trifluorobenzyl)formamido)ethyl)-6-chloro-1*H*-indole-2-carboxylic acid (18l): yellowish solid (36 mg, 93%). HPLC/MS: t_R = 12.16 min; m/z = 495.7 [M+H]$^+$. HRMS: $C_{23}H_{21}ClF_3N_3O_4Na$, 518.1070 (calcd.), 518.1069 (found). ^{1}H NMR (400 MHz, CD$_3$OD, a mixture of rotamers): 1.27 (s, 9H), 1.31 (s, 5H), 4.48 (ABd, 1H, J = 16.8 Hz), 4.57 (ABd, 1H, J = 16.0 Hz), 4.68 (ABd, 1H, J = 16.8 Hz), 4.97 (m, 1H), 6.30 (s, 1H), 6.40 (m, 1H), 6.69-6.74 (m, 1H), 6.79-6.96 (m, 2H), 7.07-7.12 (m, 2H), 7.41 (m, 2H), 7.77 (m, 1H), 7.82 (m, 1H), 8.38 (s, 1H), 8.45 (s, 1H). ^{13}C NMR (100 MHz, CD$_3$OD, a mixture of rotamers): 27.4, 27.5, 39.7, 43.7, 51.2, 52.7, 57.0, 104.1, 104.3, 104.4, 104.6, 111.7, 111.8, 112.3, 113.8, 116.8, 117.0, 121.1, 121.2, 121.7, 121.9, 124.5, 125.3, 127.9, 128.5, 130.6, 136.2, 136.3, 162.1, 162.5, 165.0, 165.4, 169.4, 169.6.

3-(2-(*tert*-Butylamino)-2-oxo-1-(*N*-(3,4,5-trifluorobenzyl)formamido)ethyl)-6-chloro-1*H*-indole-2-carboxylic acid (18m): yellowish solid (65 mg, 95%). HPLC/MS: t_R = 111.59 min; m/z = 495.7 [M+H]$^+$. HRMS: $C_{23}H_{22}ClF_3N_3O_4$, 496.1251 (calcd.), 496.1249 (found). ^{1}H NMR (600 MHz, CD$_3$OD, a mixture of rotamers): 1.17 (s, 9H), 1.19 (s, 4H), 4.10 (ABd, 1H, J = 15.6 Hz), 4.16 (ABd, 1H, J = 16.2 Hz), 4.56 (ABd, 1H, J = 16.8 Hz), 4.97 (ABd, 1H, J = 15.6 Hz), 6.07 (m, 1H), 6.19 (m, 1H), 6.32-6.35 (m, 2H), 6.65 (s, 1H), 7.02-7.04 (m, 2H), 7.30-7.32 (m, 2H), 7.50 (s, 1H), 7.55 (s, 1H), 7.67 (m, 1H), 7.69 (m, 1H), 8.28 (s, 1H), 8.33 (s, 1H). ^{13}C NMR (150 MHz, CD$_3$OD, a mixture of rotamers): 28.85, 28.94, 47.1, 49.5, 50.5, 52.8, 53.4, 54.0, 58.3, 110.8, 110.9, 112.2, 112.3, 112.4, 113.2, 113.3, 113.8, 115.2, 122.6, 122.7, 123.0, 123.2, 126.1, 126.8, 131.9, 132.0, 135.9, 137.6, 137.7, 138.6, 140.2, 150.7, 152.3, 166.1, 166.8, 171.1.

3-(2-(*tert*-Butylamino)-2-oxo-1-(*N*-(2,3,6-trifluorobenzyl)formamido)ethyl)-6-chloro-1*H*-indole-2-carboxylic acid (18n): yellowish solid (15 mg, 79%). HPLC/MS: t_R = 11.19 min; *m/z* = 495.9 [M+H]$^+$. HRMS: $C_{23}H_{21}ClF_3N_3O_4Na$, 518.1070 (calcd.), 518.1078 (found). ^{1}H NMR (600 MHz, CD$_3$OD, a mixture of rotamers): 1.20 (s, 9H), 1.31 (s, 7H), 4.63 (ABd, 1H, *J* = 15.6 Hz), 4.76 (ABd, 1H, *J* = 16.2 Hz), 4.93-5.02 (m, 2H), 6.15 (s, 1H), 6.57 (s, 1H), 6.86 (m, 1H), 6.96 (m, 1H), 7.04-7.08 (m, 2H), 7.17-7.20 (m, 2H), 7.41-7.45 (m, 2H), 7.74-7.80 (m, 2H), 8.12 (s, 1H), 8.52 (s, 1H). ^{13}C NMR (150 MHz, CD$_3$OD, a mixture of rotamers): 27.4, 27.5, 34.8, 39.5, 51.2, 53.4, 56.7, 110.5, 111.5, 111.8, 113.4, 116.5, 120.8, 121.1, 121.7, 122.0, 124.7, 125.6, 130.4, 136.2, 136.4, 164.4, 165.3, 169.0, 169.6.

3-(2-(*tert*-Butylamino)-2-oxo-1-(*N*-(2,4,6-trifluorobenzyl)formamido)ethyl)-6-chloro-1*H*-indole-2-carboxylic acid (18o): white solid (42 mg, 74%). HPLC/MS: t_R = 11.57 min; *m/z* = 495.4 [M+H]$^+$. HRMS: $C_{23}H_{22}ClF_3N_3O_4$, 496.1251 (calcd.), 496.1260 (found). ^{1}H NMR (400 MHz, CD$_3$OD, a mixture of rotamers): 1.20 (s, 9H), 1.30 (s, 5H), 4.55 (ABd, 1H, *J* = 16.0 Hz), 4.68 (ABd, 1H, *J* = 16.0 Hz), 4.83-4.93 (m, 2H), 6.09 (s, 1H), 6.44 (m, 1H), 6.55 (s, 1H), 6.75-6.79 (m, 2H), 7.03-7.08 (m, 2H), 7.42-7.47 (m, 2H), 7.74-7.78 (m, 2H), 8.08 (s, 1H), 8.49 (s, 1H). ^{13}C NMR (100 MHz, CD$_3$OD, a mixture of rotamers): 27.4, 27.5, 34.2, 39.0, 51.0, 51.1, 53.5, 56.5, 99.4, 99.7, 99.9, 111.5, 111.8, 111.9, 113.4, 120.8, 121.1, 121.8, 122.1, 124.8, 125.6, 130.4, 130.5, 136.2, 136.5, 164.4, 168.9, 169.5.

3-(2-(*tert*-Butylamino)-2-oxo-1-(*N*-(2,3,5-trifluorobenzyl)formamido)ethyl)-6-chloro-1*H*-indole-2-carboxylic acid (18p): white solid (28 mg, 60%). HPLC/MS: t_R = 11.96 min; *m/z* = 495.4 [M+H]$^+$. HRMS: $C_{23}H_{21}ClF_3N_3O_4Na$, 518.1070 (calcd.), 518.0989 (found). ^{1}H NMR (600 MHz, CD$_3$OD, a mixture of rotamers): 1.28 (s, 9H), 1.32 (s, 5H), 4.56 (ABd, 1H, *J* = 15.6 Hz), 4.63 (ABd, 1H, *J* = 16.2 Hz), 4.75 (ABd, 1H, *J* = 16.8 Hz), 5.03 (ABd, 1H, *J* = 15.6 Hz), 6.11

(m, 1H), 6.34 (s, 1H), 6.58 (m, 1H), 6.75-6.86 (m, 2H), 7.09 (m, 1H), 7.41 (s, 1H), 7.71 (s, 1H), 7.77 (d, 1H, J = 9.0 Hz), 7.82 (d, 1H, J = 9.0 Hz), 8.40 (s, 1H), 8.47 (s, 1H). ^{13}C NMR (150 MHz, CD$_3$OD, a mixture of rotamers): 27.4, 27.5, 40.0, 43.9, 51.3, 52.7, 57.0, 103.1, 103.3, 103.5, 110.2, 110.4, 111.7, 111.8, 112.1, 113.6, 121.1, 121.2, 121.5, 121.8, 124.5, 125.3, 127.6, 127.7, 130.5, 136.1, 136.2, 165.0, 165.4, 169.5, 169.6.

3-(2-(*tert*-Butylamino)-2-oxo-1-(*N*-(2,3,5,6-tetrafluorobenzyl)formamido)ethyl)-6-chloro-1*H*-indole-2-carboxylic acid (18q): white solid (22 mg, 93%). HPLC/MS: t_R = 11.44 min; m/z = 513.8 [M+H]$^+$. HRMS: C$_{23}$H$_{20}$ClF$_4$N$_3$O$_4$Na, 536.85898 (calcd.), 537.3983 (found). ^{1}H NMR (400 MHz, CD$_3$OD, a mixture of rotamers): 1.20 (s, 9H), 1.28 (s, 5H), 4.65 (ABd, 1H, J = 16.4 Hz), 4.78 (ABd, 1H, J = 16.0 Hz), 4.92-5.02 (m, 2H), 6.15 (s, 1H), 6.55 (s, 1H), 7.01-7.06 (m, 2H), 7.20-7.29 (m, 2H), 7.39-7.43 (m, 2H), 7.51 (s, 1H), 7.70-7.73 (m, 2H), 7.79 (s, 1H), 8.11 (s, 1H), 8.50 (s, 1H). ^{13}C NMR (100 MHz, CD$_3$OD, a mixture of rotamers): 27.4, 27.5, 34.8, 39.6, 51.1, 53.2, 56.9, 104.6, 105.1, 105.3, 105.6, 111.5, 111.7, 111.8, 113.1, 115.7, 121.0, 121.2, 121.6, 121.9, 124.7, 125.5, 130.4, 130.5, 136.1, 136.3, 164.5, 165.3, 168.9, 169.4.

3-(2-(*tert*-Butylamino)-2-oxo-1-(*N*-(2,3,4,6-tetrafluorobenzyl)formamido)ethyl)-6-chloro-1*H*-indole-2-carboxylic acid (18r): white solid (38 mg, 73%). HPLC/MS: t_R = 11.93 min; m/z = 513.6 [M+H]$^+$. HRMS: C$_{23}$H$_{20}$ClF$_4$N$_3$O$_4$K, 552.0716 (calcd.), 552.0752 (found). ^{1}H NMR (400 MHz, CD$_3$OD, a mixture of rotamers): 1.22 (s, 9H), 1.30 (s, 5H), 4.58 (ABd, 1H, J = 16.0 Hz), 4.73 (ABd, 1H, J = 16.0 Hz), 4.91 (m, 2H), 6.08 (s, 1H), 6.55 (s, 1H), 6.61-6.68 (m, 1H), 6.93-7.00 (m, 1H), 7.04-7.09 (m, 2H), 7.43-7.47 (m, 2H), 7.74-7.77 (m, 2H), 7.82 (s, 1H), 8.09 (s, 1H), 8.51 (s, 1H). ^{13}C NMR (100 MHz, CD$_3$OD, a mixture of rotamers): 27.4, 27.5, 31.3, 34.3, 39.2, 51.1, 51.2, 53.3, 56.5, 111.6, 111.8, 120.0, 113.5, 120.9, 121.2, 121.7, 122.0, 124.7, 125.5, 127.7, 130.55, 130.61, 136.2, 136.4, 164.4, 165.2, 168.86, 168.94, 169.4.

3-(2-(*tert*-Butylamino)-2-oxo-1-(*N*-(2,3,4,5-tetrafluorobenzyl)formamido)ethyl)-6-chloro-1*H*-indole-2-carboxylic acid (18s): white solid (27 mg, 84%). HPLC/MS: t_R = 11.36 min; m/z = 441.8 [M+H]$^+$. HRMS: $C_{23}H_{20}ClF_4N_3O_4Na$, 536.85898 (calcd.), 537.3737 (found). ^{1}H NMR (600 MHz, CD$_3$OD, a mixture of rotamers): 1.22 (s, 9H), 1.31 (s, 6H), 4.30-4.35 (m, 2H), 4.71 (ABd, 1H, J = 16.2 Hz), 5.14 (ABd, 1H, J = 15.0 Hz), 6.27 (s, 1H), 6.52 (m, 1H), 6.80 (s, 1H), 6.92-6.95 (m, 3H), 7.05-7.13 (m, 5H), 7.37-7.47 (m, 4H), 7.80 (d, 1H, J = 9.0 Hz), 7.85 (d, 1H, J = 9.0 Hz), 8.39 (s, 1H), 8.45 (s, 1H). ^{13}C NMR (150 MHz, CD$_3$OD, a mixture of rotamers): 27.4, 27.5, 47.0, 51.1, 51.2, 52.0, 52.8, 56.9, 111.7, 111.8, 112.5, 114.0, 120.8, 121.0, 121.8, 122.0, 124.8, 125.3, 125.6, 126.5, 127.3, 130.2, 136.3, 136.8, 137.5, 162.6, 164.7, 165.3, 169.6, 169.9.

3-(2-(*tert*-Butylamino)-2-oxo-1-(*N*-(perfluorobenzyl)formamido)ethyl)-6-chloro-1*H*-indole-2-carboxylic acid (18t): white solid (55 mg, 92%). HPLC/MS: t_R = 12.18 min; m/z = 531.7 [M+H]$^+$. HRMS: $C_{23}H_{19}ClF_5N_3O_4K$, 570.0621 (calcd.), 570.0630 (found). ^{1}H NMR (400 MHz, CD$_3$OD, a mixture of rotamers): 1.25 (s, 9H), 1.31 (s, 6H), 4.63 (ABd, 1H, J = 16.0 Hz), 4.79 (ABd, 1H, J = 16.0 Hz), 4.90-5.01 (m, 2H), 6.10 (s, 1H), 6.55 (s, 1H), 7.06-7.11 (m, 2H), 7.43-7.47 (m, 2H), 7.73-7.76 (m, 2H), 8.11 (s, 1H), 8.52 (s, 1H). ^{13}C NMR (100 MHz, CD$_3$OD, a mixture of rotamers): 27.3, 27.5, 34.1, 39.3, 51.1, 51.2, 53.2, 56.5, 111.5, 111.8, 113.1, 121.0, 121.2, 121.6, 121.9, 124.7, 125.5, 130.5, 130.6, 136.1, 136.3, 164.4, 168.9.

2.4   DISCOVERY AND EVALUATION OF P53-MDM2 INHIBITORS AS POTENTIAL ANTICANCER DRUG CANDIDATES

Inspried by the detail 3D-structural information of small molecule/Mdm2 complexes, we intended to discover p53-Mdm2 inhibitors as potential anticancer drug candidates from structure-

based drug design to preclinical proof-of-concept studies. Imidazole-indole scaffold-based compounds were previously identified as potent inhibitors bound to Mdm2 and Mdm4.[92, 162] As a bioisostere of imidazole, pyrazole is a privileged scaffold for the generation of new drugs (**Figure 19**), such as Celecoxib (SC-58635) and Rimonabant (SR141716).[163, 164] Although pyrazole and imidazole are positional isomers with nearly identical structure and perhaps similar interactions with their targets, they have shown different pharmacological and pharmacokinetic profiles.[165] In order to further optimize their biochemical and physicochemical properties, p53-Mdm2 inhibitors based on a pyrazole-indole scaffold were developed.[166]
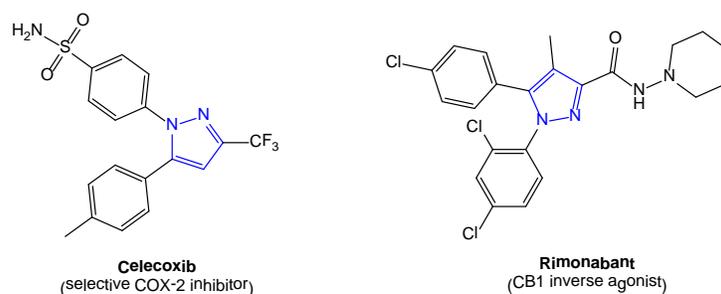
Figure 19. Examples of drugs possessing a pyrazole moiety

Dimethylformamide dimethyl acetal (DMF-DMA) and its analogs can server as bulding blocks for the synthesis of heterocycles, including pyrazoles.[167] The compounds based on pyrazole-indole scaffold were synthesized via an "one-pot" reaction: **19a-b** condensed with dimethylformamide diethyl acetal (DMF-DEA) to give the corresponding enamines, which were subsequently treated with benzyl hydrazine hydrochloride **20a-d** to provide pyrazoles **21** (**Scheme 7**). The ester group of **21a-f** was saponified to give the corresponding acid compounds **22a-f**, since the 2-carboxylic acid moiety of the indole ring is known to improve the binding affinity with Mdm2.
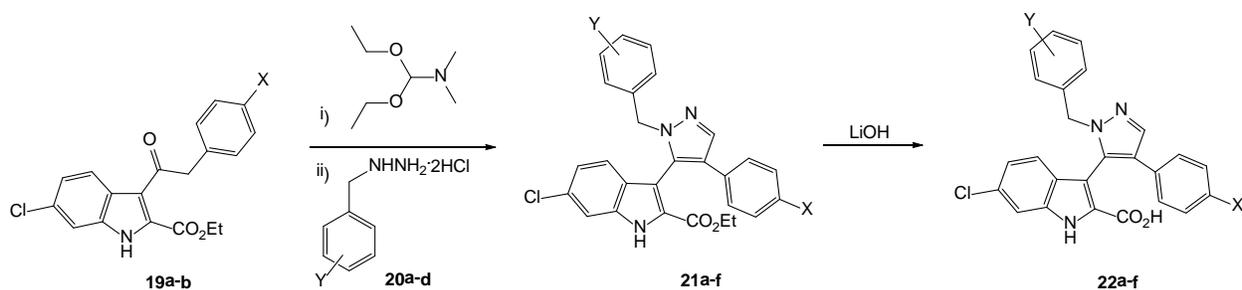
Scheme 7. Synthesis of pyrazoles

All the compounds were subjected to primary screening via the FP assay. **Table 7** summarizes the structure-activity study of these compounds. The acid compounds **22a-f** show improved potency compared with the corresponding parent ethyl ester compounds **21a-f**. The chlorine substitution at the Y position improves the binding affinity to Mdm2, which can be explained by the favorable interaction between the imidazole isomer and Leu26 binding pocket, as shown in the cocrystal structure of PB12/Mdm2 complex (**Figure 8**, **Chapter 2.2.1**). The fluorine substitution at the X position is also favorable to Phe19 binding pocket, as shown in a recently resolved cocrystal structure of WK/Mdm2 complex.[168] Consistently, **22c** was identified as the most potent compound (Ki = 100 nM) in this series.

Table 7. Inhibition constants [µM] of pyrazole compounds.

No.	X	hydrazine	K_i (**21**) [a]	K_i (**22**) [a]
a	H		n.i.	1.4
b	H		30	0.3
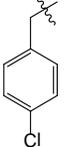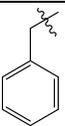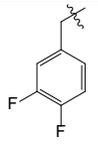
c	F		N.A.	0.1
d	F		n.i.	4
e	F		n.i.	0.8
f	F		10	0.35
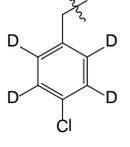
[a]Measured by fluorescent polarization assay.

It has been shown that the transformation of the carboxylic acid group of the indole fragment to an amide group does not affect the binding mode, and hopefully can improve the binding affinity to Mdm2.[92] A side chain was introduced to compounds **22b-c**, which were selected according to their promising binding affinities. Compounds **24b-c** were then produced (**Scheme 8**), and screened against Mdm2 via the FP assay. Compound **24c** showed the potent binding affinity with Ki as low as 20 nM.
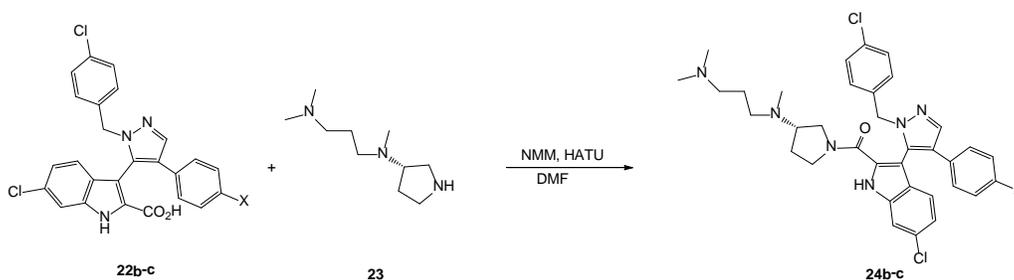
Scheme 8. Synthesis of tagged pyrazoles

Compounds were further subjected to secondary screening via a biosensor assay to identify the cellular activity of p53-Mdm2 inhibitors.[169] p53 and Mdm2 were expressed as green fluorescent protein (GFP) and red fluorescent protein (RFP) constructs, respectively. Mdm2 and p53 components of the biosensor resulted in both proteins becoming localized to the nucleolus, producing a yellow signal in composite images. Upon disruption of the p53-Mdm2 protein-protein interaction with a compound such as nutlin-3, the p53-GFP interaction partner remained nucleolar, while the shuttling Mdm2-RFP interaction partner redistributed into the cytoplasm. Thus, the nucleolus was predominantly light green/blue and the cytoplasm was predominantly red after treatment with p53-Mdm2 inhibitors (**Figure 21**). For the determination of the 50% inhibition concentrations (IC_{50}), 10-point 2-fold serial dilutions of compounds were tested (**Fiure 20**). Compound **24c** (IC_{50} = 1.8 µM) has showed efficacy comparable to the reference compound, nutlin-3 (IC_{50} = 2 µM).
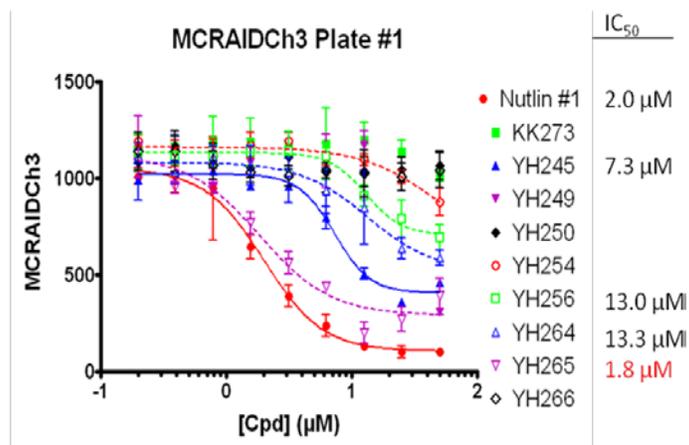
Figure 20. IC_{50} determinations (**24c** = YH265).
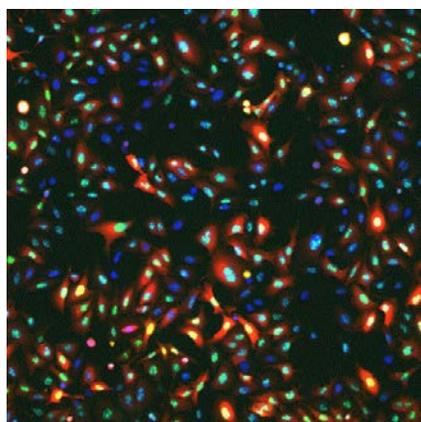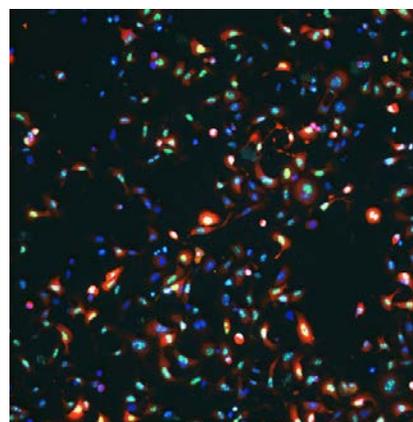
6 µM Nutlin-3a 6 µM YH265

Figure 21. Fluorescent imaging of U-2 OS cells coinfected with both the p53-GFP and Mdm2-RFP adenoviruses.

A selection of compounds were submitted to the NCI for testing their ability to arrest the growth of cancer cells in 60 different cancer cell lines.[170] For example, a single 10 µM dose of the test compound was added to each well of the cell culture plate as a primary screening assay. The cell density and viability was measured, and the percent inhibition for each cell line was determined (**Appendix D**). The selected compounds were further evaluated against 60 cancer cell lines using different concentrations in order to determine the 50% growth inhibitory concentrations (GI_{50}). **Figure 22** summarizes the GI_{50} (µM) values for a selection of compounds. Consistently, compound **24c** (GI_{50} = 4.42 µM) has shown comparable efficacy with the reference compound, nutlin-3 (GI_{50} = 2.37 µM).

Color legend

| 1.00 | 2.00 | 3.00 | 4.00 | 5.00 | 6.00 | 7.00 | 8.00 | 9.00 | 10.00 |

PANELNME: Leukemia

CELLNAME	CCRF-CEM	HL-60(TB)	K-562	MOLT-4	RPMI-8226	SR	Average
YH263	4.03	2.91	2.58	3.05	3.70	3.56	3.31
YH265	4.61	6.03	0.67	5.28	4.52	5.73	4.47
Nutlin-3a	3.85	3.62	3.47	3.39	3.58	2.33	3.37
WW528	1.80	1.88	1.45	1.69	1.95	1.80	1.76
WW515	10.30	9.10	4.63	7.25	5.22	9.68	7.70

Non-Small Cell Lung Cancer

CELLNAME	A549/ATC	EKVX	HOP-62	HOP-92	NCI-H226	NCI-H23	NCI-H322	NCI-H460	NCI-H522	Average
YH263	3.37	3.68	3.49	2.60	2.93	3.22	4.48	1.89	3.29	3.22
YH265	4.79	4.82	4.31	1.74	4.42	4.69	4.92	3.84	5.88	4.38
Nutlin-3a	2.22	2.08	1.90	2.10	2.08	1.83	1.99	1.95	2.09	2.03
WW528	1.84		1.49	1.64	1.74	1.80	1.82	1.84	1.74	1.74
WW515	6.64	3.17	1.04	1.98	5.63	7.03	9.18	4.42	5.36	4.94

Colon Cancer

CELLNAME	COLO 205	HCC-2998	HCT-116	HCT-15	HT29	KM12	SW-620	Average
YH263	2.58	4.59	2.96	3.56	3.14	3.60	3.77	3.46
YH265	1.60	3.83	2.77	4.64	1.62	4.26	5.06	3.40
Nutlin-3a	3.31	2.30	1.81	1.91	3.27	1.84	2.41	2.41
WW528	1.64	1.86	1.92	1.87	1.74	1.70	1.71	1.78
WW515	7.01	8.02	2.85	7.82	4.45	7.25	7.40	6.40

PANELNME: CNS Cancer

CELLNAME	SF-268	SF-295	SF-539	SNB-19	SNB-75	U251	Average
YH263	3.63	2.44	5.47	5.45	3.69	4.10	4.13
YH265	4.31	5.50	5.67	5.48	4.56	4.17	4.95
Nutlin-3a	2.11	1.81	2.02	2.08	2.37	1.84	2.04
WW528	1.65		1.72	1.80	1.65	1.61	1.69
WW515	7.49	2.67	1.06	1.41	7.57	8.54	4.79

Melanoma

CELLNAME	LOXIMVI	MALME-3M	M14	MDA-MB	SK-MEL-2	SK-MEL-28	SK-MEL-5	UACC-257	UACC-62	Average
YH263	3.21	3.66	3.58	3.49	4.42	7.40	2.92	3.84	3.89	4.05
YH265	4.42	5.54	5.49	2.58	6.49	5.62	4.35	5.01	5.10	4.96
Nutlin-3a	1.78		1.84	1.92	2.08	3.24	1.76	2.64	1.90	2.15
WW528	1.74	1.94	2.08		2.17	1.87	1.62	1.82	1.90	1.89
WW515	5.93	7.71	6.24	6.37	6.94	8.34	6.06	7.96	4.26	6.65

CELLNAME	IGROV1	OVCAR-3	OVCAR-4	OVCAR-5	OVCAR-8	NCI/ADR-15K-OV-3	Average
YH263	4.13	3.39	4.61	3.58	4.03	3.48	5.30	4.07
YH265	4.47	3.85	3.61	5.75	5.44	5.33	4.76	4.74
Nutlin-3a	1.76	1.71	2.16	2.23	2.46	1.92	2.27	2.07
WW528	1.84	1.70		1.68	1.76	2.05	1.68	1.79
WW515	6.69	6.23	1.04	7.74	9.12	6.34	1.38	5.51

PANELNME: Renal Cancer

CELLNAME	786-0	A498	ACHN	CAKI-1	RXF 393	SN12C	TK-10	UO-31	Average
YH263	5.65	2.55	3.02	1.62	2.61	2.46		1.80	
YH265	6.06	4.39	5.56	5.07	4.25	5.42		3.67	
Nutlin-3a		1.89	1.89	1.80	3.60	1.94	1.93		
WW528	2.08	1.59	1.81		1.62	1.77			
WW515	7.62	5.71	7.35	2.03	5.60	5.45		2.31	

Prostate Cancer / Breast Cancer

CELLNAME	PC-3	Average	DU-145	MCF7	MDA-MB	HS 578T	BT-549	T-47D	MDA-MB	Average
YH263	2.94	2.83	3.77	3.49	2.98		1.02	3.05	2.76	2.85
YH265	4.23	4.83	4.32	3.13	4.39		5.09	5.32	4.28	4.42
Nutlin-3a	2.26	2.19	1.80	2.06	1.98	2.65	2.03	3.67		2.37
WW528	1.41	1.67	1.71	1.64	1.80	1.73	1.91	1.94	1.61	1.77
WW515	4.26	5.04	9.66	5.94	3.14		9.48	3.89	5.34	6.24

Figure 22. GI_{50} (µM) values for a selection of compounds

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The aqueous solubility is one of the most important properties for drug discovery, and a determinant of intestinal absorption and oral bioavailability.[171] Since p53-Mdm2 inhibitors target a hydrophobic protein-protein interface, they are very hydrophobic in general (cLogP > 5 for pyrazole compounds). The aqueous solubility of the potential candidates for pre-clinical studies was measured using the shake-flask method (**Appendix C**). **Table 8** illustrates the solubility of pyrazole compounds in 10 mM Na_3PO_4 buffer (pH = 7.0, 20 ^{o}C). It shows that acid compounds have reasonable high aqueous solubility for further development.

Table 8. Aqueous solubility in a phosphate buffer

ID	Structure	Solubility
22b (YH245)		1.31 mg/mL
21c (YH263)		37.9 µg/mL
22c (YH264)		1.66 mg/mL
24c (YH265)		0.54 mg/mL
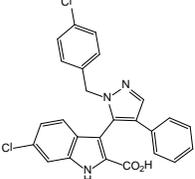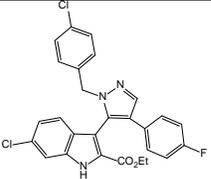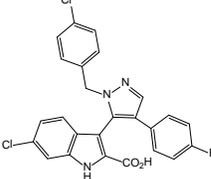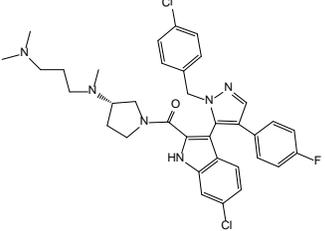
Suprisingly, compound **21c** (GI_{50} = 2.85 µM) has shown good efficacy according to the GI_{50} value measured by NCI-60 assay, which could act as the prodrug of **22c** (K_i = 100 nM against Mdm2) *in vivo*. Therefore, compound **21c** and **22c** were selected for animal studies by the University of Pittsburgh Cancer Institute. Compounds **21c** and **22c** displayed *in vitro* cytotoxicity against HCT-116 human colon carcinoma with IC_{50} values as 8.9 ± 0.6 µM and 18.6± 2.3 µM, respectively. **21c** was administrated (multidose MTD 150 mg/kg) i.v. and p.o. in C.B-17 SCID mice bearing HCT-116 xenografts. However, the tumor growth has no significant difference compared with the vehicle control (**Figure 23A**). Some metabolits of **22c** were identified in plasma (**Figure 23B**). Figure C shows the life-time pharmacokinetic study (**Figure 23C**) completed in SCID mice with HCT-116 xenografts. The further analysis shows that tumor concentration was only 1% of plasma concentration for i.v. injection, and no detectable tumor concentration was observed for oral dosing.
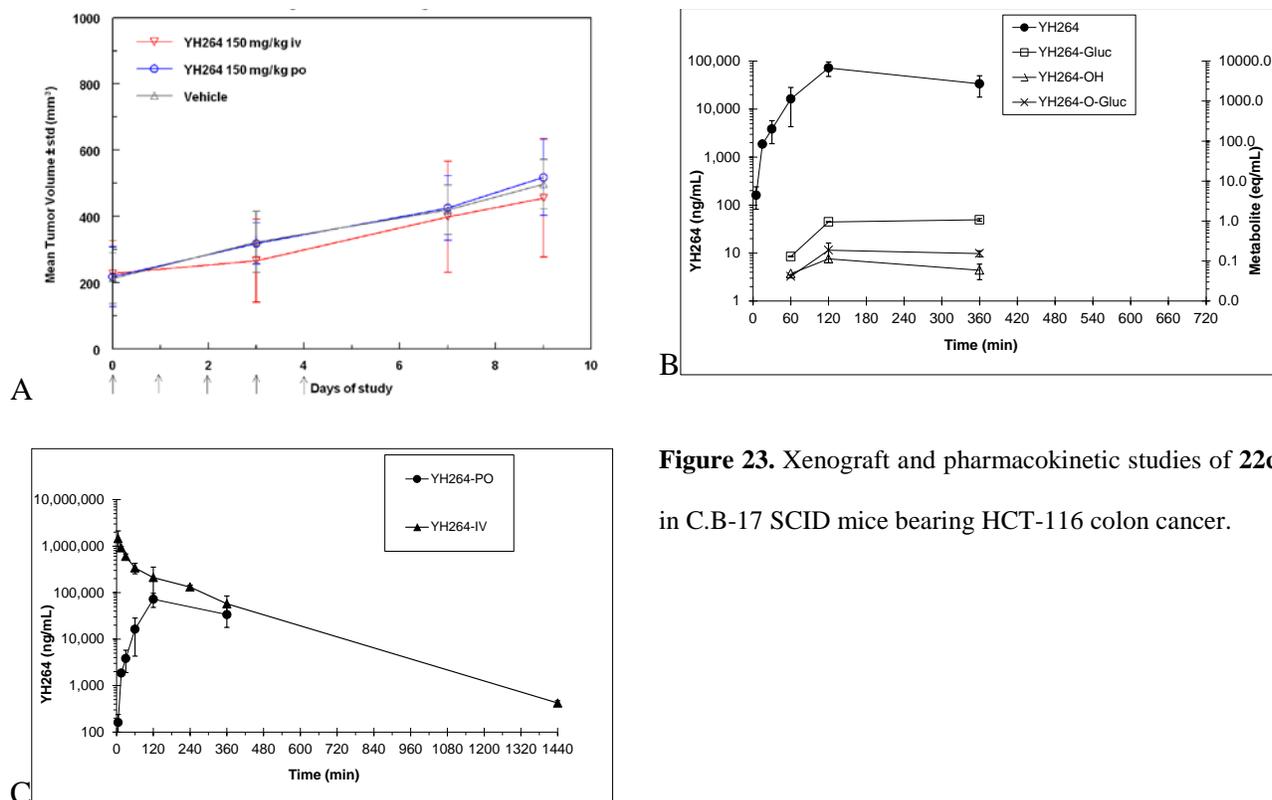
Figure 23. Xenograft and pharmacokinetic studies of **22c** in C.B-17 SCID mice bearing HCT-116 colon cancer.

The combinational therapy of p53-Mdm2 inhibitor nutlin-3 and histone deacetylase (HDAC) inhibitor valproic acid has shown better efficacy in a mouse model.[172] Since HDACs also participate in p53 deacetylation and destabilization,[173] p53-Mdm2 inhibitor and HDAC inhibitor may have synergestic antitumor activity. Therefore, it provides a new strategy to design dual functional small molecules which can inhibit p53-Mdm2 interaction and HDAC activity simultaneously. Vorinostat (SAHA) was discovered as HDAC inhibitor and approved for treatment of cutaneous T cell lymphoma (CTCL). The crystal structures of human HDAC8 complexed with four structurally diverse hydroxamate inhibitors were resolved.[174] For example, the hydroxamate functional group of SAHA binds to the zinc at the end of the hydrophobic tunnel, thus blocks the HDAC activity at the enzyme catalytic site (**Figure 24**). In order to design dual functional compounds, we intended to combine the pharmacophore of HDAC inhibitors (the long chain with the hydroxamate functional group) and the scaffold of p53-Mdm2 inhibitors.
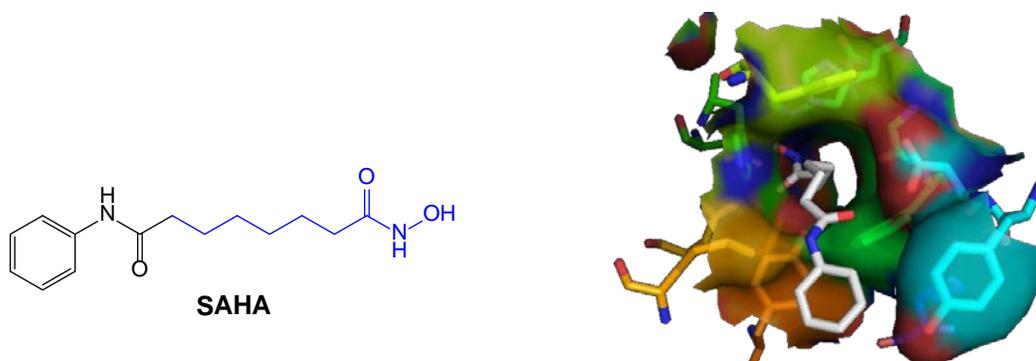
Figure 24. Crystal structure of human HDAC8 complexed with SAHA (PDB ID: 1T69).

We introduced the long chain with the hydroxamate functional group to p53-Mdm2 inhibitors as a side chain, which may not interfere with their binding model according to the known cocrystal structure.[92] The desired amino alkanoic acid derivatives were coupled with p53-Mdm2 inhibitors via amidation to give compound a, which were hydrolyzed to form compounds with the terminal carboxylic group (**Scheme 9**). Compounds were treated with hydroxylamine to

provide compounds with the hydroxamate functional group. All the compounds were measured by FP assay (**Table 9**). Compounds **26** and **27** have shown reasonable binding affinities to Mdm2, and the chain length doesn't interfere with their binding model. These compounds were evaluated by collarborators in Leiden University Medical Center.
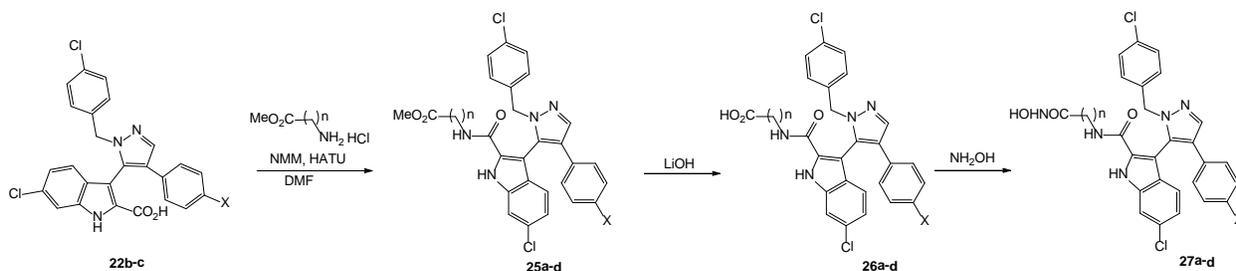
Scheme 9. Modification of pyrazoles

Table 9. Inhibition constants [μM] of tagged compounds.

entry	X	n	K_i (**25**) [a]	K_i (**26**) [a]	K_i (**27**) [a]
a	H	6	n.i.	0.4	1.2
b	F	6	N.A.	0.6	1.3
c	F	5	20	0.9	0.8
d	F	7	55	1.2	2

[a]Measured by fluorescent polarization assay.

In summary, we have shown new directions for the optimization of potent p53-Mdm2 inhibitors as potential anticancer drug candidates. Novel and potent agents targeting p53-Mdm2 interaction are still needed for desirable anti-tumor efficacy in vivo.

Materials and Methods

Column chromatography was performed using SiO_2 60 Å (particle size 0.040-0.055 mm, 230-400 mesh, EM science distributed by Bioman).

General procedure for the synthesis of compounds 21:

Preparation of **ethyl 6-chloro-3-(1-(4-chlorobenzyl)-4-phenyl-1*H*-pyrazol-5-yl)-1*H*-indole-2-carboxylate (21b, Method A):** The mixture of ethyl 6-chloro-3-(2-phenylacetyl)-1H-indole-2-carboxylate (5 mmol, 1.70 g) in 2.5 mL of dimethylformamide diethyl acetal (DMF-DEA) was stirring under 50 $^{\circ}$C for 1 hour. Cooled to room temperature, the solvent was evaporated in vacco. The residue was treated with 4-chlorobenzyl hydrazine hydrochloride (1.15 g, 5 mmol), and 10 mL of ethanol. The mixture was kept under reflux overnight. Cooled to room temperature, the solvent was evaporated in vacco. The product was purified by column chromatography on silica gel (petroleum ether/ethyl acetate, 5:1) as yellow solid (0.43 g, yield: 18%). HPLC/MS: t_R = 19.47 min; m/z = 490.1 [M+H]$^+$. HRMS: $C_{27}H_{22}Cl_2N_3O_2$, 490.1089 (calcd.), 490.1062 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 9.59 (s, 1H), 7.94 (s, 1H), 7.49 (1H, s), 7.10-7.19 (m, 8H), 6.85 (d, 2H, J = 7.8 Hz), 5.16 (1H, ABd, J = 15.6 Hz), 5.00 (1H, ABd, J = 15.0 Hz), 3.97-4.11 (m, 2H), 1.05 (t, 3H, J = 7.2 Hz). ^{13}C NMR (150 MHz, CDCl$_3$): 160.9, 138.0, 136.0, 135.2, 133.5, 132.9, 132.3, 131.4, 128.9, 128.5, 128.4, 126.6, 126.4, 126.2, 123.4, 123.0, 122.2, 112.1, 110.6, 61.4, 53.2, 13.8.

Preparation of **ethyl 3-(1-benzyl-4-phenyl-1*H*-pyrazol-5-yl)-6-chloro-1*H*-indole-2-carboxylate (21a, Method A):** The product was purified by column chromatography on silica gel (petroleum ether/ethyl acetate, 5:1) as yellow solid (6 mg, yield: 18%). HPLC/MS: t_R = 12.65 min; m/z = 456.3 [M+H]$^+$. HRMS: $C_{27}H_{23}ClN_3O_2$, 456.1479 (calcd.), 456.1486 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 9.35 (s, 1H), 7.93 (s, 1H), 7.49 (1H, s), 7.08-7.21 (m, 9H), 6.91 (m, 2H), 5.21 (1H, ABd, J = 15.0 Hz), 5.02 (1H, ABd, J = 15.6 Hz), 3.90-4.07 (m, 2H), 1.02 (t, 3H, J = 7.2 Hz). ^{13}C NMR (150 MHz, CDCl$_3$): 13.7, 54.0, 61.3, 110.8, 112.0, 122.3, 123.0, 123.2,

126.1, 126.3, 126.5, 126.7, 127.5, 127.6, 128.3, 128.4, 131.4, 132.2, 133.1, 135.9, 136.7, 137.8, 161.0.

Preparation of **ethyl 6-chloro-3-(1-(4-chlorobenzyl)-4-(4-fluorophenyl)-1*H*-pyrazol-5-yl)-1*H*-indole-2-carboxylate (21c, Method A):** The product was purified by column chromatography on silica gel (petroleum ether/ethyl acetate, 5:1) as yellow solid (0.76 g, yield: 30%). HPLC/MS: t_R = 12.59 min; m/z = 508.2 [M+H]$^+$. HRMS: $C_{27}H_{20}N_3O_2FCl_2$, 507.091661 (calcd.), 507.092044 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.07 (t, 3H, J = 7.2 Hz), 3.99-4.13 (m, 2H), 5.00 (1H, ABd, J = 15.0 Hz), 5.16 (1H, ABd, J = 15.6 Hz), 6.83-6.86 (m, 4H), 7.09-7.17 (m, 6H), 7.51 (s, 1H), 7.88 (s, 1H), 9.30 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 13.9, 53.3, 61.4, 110.4, 112.1, 115.3, 115.5, 122.1, 122.5, 123.1, 126.3, 126.6, 127.9, 128.4, 128.9, 129.0, 131.3, 132.5, 133.5, 135.2, 135.9, 137.8, 160.6, 162.2.

Preparation of **ethyl 3-(1-benzyl-4-(4-fluorophenyl)-1*H*-pyrazol-5-yl)-6-chloro-1*H*-indole-2-carboxylate (21d, Method A):** The product was purified by column chromatography on silica gel (petroleum ether/ethyl acetate, 5:1) as yellow solid (114 mg, yield: 48%). HRMS: $C_{27}H_{22}ClFN_3O_2$, 474.1385 (calcd.), 474.1388 (found). ^{1}H NMR (400 MHz, CDCl$_3$): 1.05-1.08 (t, 3H, J = 7.2 Hz), 3.97-4.16 (m, 2H), 5.04 (ABd, 1H, J = 14.8 Hz), 5.25 (ABd, 1H, J = 15.2 Hz), 6.82-6.87 (m, 2H), 6.92-6.94 (m, 2H), 7.10-7.16 (m, 6H), 7.22 (m, 1H), 7.49 (s, 1H), 7.94 (s, 1H). ^{13}C NMR (100 MHz, CDCl$_3$): 13.8, 54.0, 61.4, 110.5, 112.3, 115.3, 115.5, 122.1, 122.4, 123.0, 126.4, 126.7, 127.5, 127.6, 127.9, 128.0, 128.3, 129.2, 129.3, 131.6, 132.2, 136.4, 136.7, 137.6, 160.2, 161.3, 162.6.

Preparation of **ethyl 6-chloro-3-(1-(3,4-difluorobenzyl)-4-(4-fluorophenyl)-1*H*-pyrazol-5-yl)-1*H*-indole-2-carboxylate (21e, Method A):** The product was purified by column chromatography on silica gel (petroleum ether/ethyl acetate, 5:1) as yellow solid (42 mg, yield:

17%). SFC/MS: t_R = 3.16 min; m/z = 510.15 [M+H]$^+$. HRMS: $C_{27}H_{20}ClF_3N_3O_2$, 510.1196 (calcd.), 510.1254 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.00 (t, 3H, J = 7.2 Hz), 3.95-4.06 (m, 2H), 4.90 (ABd, 1H, J = 15.6 Hz), 5.04 (ABd, 1H, J = 15.0 Hz), 6.51-6.53 (m, 1H), 6.66-6.84 (m, 4H), 7.00-7.09 (m, 5H), 7.42 (s, 1H), 7.80 (s, 1H), 9.31 (br.s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 13.8, 52.8, 61.5, 110.0, 110.2, 112.2, 115.3, 115.5, 116.4, 116.5, 117.0, 117.1, 121.9, 122.6, 123.2, 126.2, 126.5, 127.9, 132.5, 135.9, 138.0, 160.6.

Preparation of **ethyl 6-chloro-3-(1-(4-chlorobenzyl-2,3,5,6-d$_4$)-4-(4-fluorophenyl)-1H-pyrazol-5-yl)-1H-indole-2-carboxylate (21f, Method A)**: The product was purified by column chromatography on silica gel (petroleum ether/ethyl acetate, 3:1) as yellow solid (60 mg, yield: 17%). HPLC/MS: t_R = 12.73 min; m/z = 511.7 [M+H]$^+$. HRMS: $C_{27}H_{17}D_4Cl_2FN_3O_2$, 512.1246 (calcd.), 512.1270 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.07 (t, 3H, J = 7.2 Hz), 4.05-4.08 (m, 2H), 6.84-6.87 (m, 2H), 7.09-7.12 (m, 3H), 7.45-7.47 (m, 2H), 7.65 (s, 1H), 9.15 (br.s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 13.9, 55.5, 61.0, 111.5, 114.6, 115.3, 115.4, 122.3, 122.8, 123.1, 125.5, 127.1, 127.7, 128.1, 128.2, 129.2, 131.8, 134.9, 135.9, 141.9, 161.2, 162.3.

General procedure for the synthesis of compounds 22:

Preparation of **6-chloro-3-(1-(4-chlorobenzyl)-4-phenyl-1H-pyrazol-5-yl)-1H-indole-2-carboxylic acid (22b, Method B):** The mixture of **21b** (120 mg, 0.23 mmol), THF (1 mL), water (1 mL), LiOH (35 mg) was stirring under RT for 2 days. The reaction mixture was acidified with 1M HCl (pH ~ 6). Then the mixture was extracted with DCM (30 mL x 3). The combined organic layer was dried over sodium sulfate, and evaporated. After removal of the solvents, 102 mg of yellow solid (90%) was obtained. HPLC/MS: t_R = 18.60 min; m/z = 462.1 [M+H]$^+$. HRMS: $C_{25}H_{17}Cl_2N_3O_2$, 461.069782 (calcd.), 461.069688 (found). ^{1}H NMR (600 MHz, CD$_3$OD): 5.00-5.08 (m, 2H), 6.73 (m, 2H), 6.77-6.81 (m, 2H), 6.93-7.01 (m, 5H), 7.07 (m, 2H),

7.39 (s, 1H), 7.83 (s, 1H). ^{13}C NMR (150 MHz, CD$_3$OD): 54.1, 113.3, 122.7, 122.9, 124.4, 127.3, 127.5, 129.4, 130.0, 132.4, 134.2, 134.3, 137.0, 138.0, 138.5.

Preparation of **3-(1-benzyl-4-phenyl-1*H*-pyrazol-5-yl)-6-chloro-1*H*-indole-2-carboxylic acid (22a, Method B):** 5 mg of yellow solid (89%) was obtained. HPLC/MS: t_R = 11.78 min; m/z = 428.1 [M+H]$^+$. HRMS: C$_{25}$H$_{17}$ClN$_3$O$_2$, [M-H]$^-$; 426.1009 (calcd.), 426.1029 (found). ^{1}H NMR (600 MHz, CD$_3$OD): 7.89 (s, 1H), 7.42 (1H, s), 7.26-7.27 (m, 2H), 7.01-7.09 (m, 6H), 6.88 (m, 2H), 6.71 (m, 2H), 5.39 (1H, ABd, J = 15.6 Hz), 5.21 (1H, ABd, J = 15.6 Hz). ^{13}C NMR (150 MHz, CD$_3$OD): 53.2, 105.7, 111.2, 120.0, 120.9, 121.9, 125.3, 126.2, 126.3, 126.7, 127.0, 127.8, 128.7, 133.4, 134.6, 135.4, 136.6, 137.3, 166.8.

Preparation of **6-chloro-3-(1-(4-chlorobenzyl)-4-(4-fluorophenyl)-1*H*-pyrazol-5-yl)-1*H*-indole-2-carboxylic acid (22c, Method B):** 135 mg of yellow solid (94%) was obtained. HPLC/MS: t_R = 12.21 min; m/z = 480.1 [M+H]$^+$. HRMS: C$_{25}$H$_{15}$Cl$_2$FN$_3$O$_2$, [M-H]$^-$; 478.0525 (calcd.), 478.0550 (found). ^{1}H NMR (400 MHz, CD$_3$OD): 5.14 (ABd, 1H, J = 14.8 Hz), 5.27 (ABd, 1H, J = 14.8 Hz), 6.71-6.79 (m, 5H), 6.96-6.98 (m, 2H), 7.21-7.24 (m, 2H), 7.39 (s, 1H), 7.86 (s, 1H). ^{13}C NMR (150 MHz, CD$_3$OD): 52.7, 111.9, 114.6, 114.8, 121.2, 121.5, 122.0, 125.9, 127.9, 128.6, 129.1, 130.9, 132.8, 132.9, 135.5, 136.5, 137.1, 160.6, 162.2.

Preparation of **3-(1-benzyl-4-(4-fluorophenyl)-1*H*-pyrazol-5-yl)-6-chloro-1*H*-indole-2-carboxylic acid (22d, Method B):** 60 mg of yellow solid (84%) was obtained. HPLC/MS: t_R = min; m/z = [M+H]$^+$. HRMS: C$_{25}$H$_{17}$ClFN$_3$O$_2$, 446.1060 (calcd.), 446.1057 (found). ^{1}H NMR (400 MHz, CD$_3$OD): 4.94-5.21 (m, 2H), 6.81-6.90 (m, 6H), 7.06 (m, 3H), 7.15-7.19 (m, 2H), 7.50 (s, 1H), 7.91 (s, 1H). ^{13}C NMR (100 MHz, CD$_3$OD): 53.3, 109.4, 111.9, 114.6, 114.8, 121.3, 121.5, 125.9, 127.0, 127.1, 127.9, 129.2, 131.0, 132.7, 136.6, 136.7, 137.0, 160.2, 162.6.

Preparation of **6-chloro-3-(1-(3,4-difluorobenzyl)-4-(4-fluorophenyl)-1*H*-pyrazol-5-yl)-1*H*-indole-2-carboxylic acid (22e, Method B):** 15 mg of yellow solid (88%) was obtained. SFC/MS: t_R = 6.29 min; m/z = 480.16 [M+H]$^+$. HRMS: $C_{25}H_{14}ClF_3N_3O_2$, 480.0727 (calcd.), 480.1767 (found). ^{1}H NMR (600 MHz, CD$_3$OD): 5.16 (ABd, 1H, J = 15.6 Hz), 5.29 (ABd, 1H, J = 15.6 Hz), 6.66-6.69 (m, 2H), 6.74-6.92 (m, 4H), 7.25-7.28 (m, 2H), 7.43 (s, 1H), 7.88 (s, 1H). ^{13}C NMR (150 MHz, CD$_3$OD): 52.2, 111.4, 114.3, 114.5, 116.0, 116.4, 116.5, 120.2, 120.4, 121.3, 123.6, 126.1, 127.9, 128.0, 128.9, 129.5, 134.5, 135.2, 135.4, 136.8, 152.3, 152.4, 152.5.

Preparation of **6-chloro-3-(1-(4-chlorobenzyl-2,3,5,6-d$_4$)-4-(4-fluorophenyl)-1*H*-pyrazol-5-yl)-1*H*-indole-2-carboxylic acid (22f, Method B)**: 45 mg of yellow solid (97%) was obtained. HPLC/MS: t_R = min; m/z = [M+H]$^+$. HRMS: $C_{25}H_{13}D_4Cl_2FN_3O_2$, 484.0933 (calcd.), 484.0909 (found). ^{1}H NMR (600 MHz, CD$_3$OD): 5.16 (ABd, 1H, J = 15.6 Hz), 5.30 (ABd, 1H, J = 15.0 Hz), 6.70 (m, 1H), 6.77-6.79 (m, 4H), 7.24-7.26 (m, 2H), 7.41 (s, 1H), 7.87 (s, 1H). ^{13}C NMR (150 MHz, CD$_3$OD): 52.5, 111.5, 114.4, 114.5, 120.3, 120.6, 121.2, 126.2, 127.9, 128.3, 129.0, 129.5, 132.4, 134.5, 135.5, 135.7, 136.8, 160.4, 162.0.

General procedure for the synthesis of compounds 24:

Preparation of **(6-chloro-3-(1-(4-chlorobenzyl)-4-phenyl-1*H*-pyrazol-5-yl)-1*H*-indol-2-yl)((*S*)-3-((3-(dimethylamino)propyl)(methyl)amino)pyrrolidin-1-yl)methanone (24b, Method C):** The mixture of **22b** (60 mg, 0.13 mmol), (*S*)-N^1,N^1,N^3-trimethyl-N3-(pyrrolidin-3-yl)propane-1,3-diamine (27.8 mg, 0.15 mmol), *N*-methylmorpholine (NMM) (50 µL, 0.45 mmol) and HATU (2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate) (65 mg, 0.17 mmol) in 0.5 mL of DMF is heated for 1 h at 50 ^{o}C. Ethylacetate is added and the organic layer is washed with brine, dried and evaporated in vacuo. The residue was purified by silica gel column chromatography (EtOAc/MeOH, 3/1; 0.5% Et$_3$N) to give the product as yellow

solid (38 mg, 49% yield). HPLC/MS: t_R = 10.73 min; m/z = 629.0 [M+H]$^+$. HRMS: $C_{35}H_{39}Cl_2N_6O$, 629.2562 (calcd.), 629.2571 (found). ^{1}H NMR (600 MHz, CD$_3$OD): 1.28 (m, 1H), 1.76-1.86 (m, 2H), 2.55 (m, 2H), 2.76-78 (m, 6H), 2.84-2.97 (m, 2H), 3.10-3.22 (m, 2H), 3.29 (m, 4H), 3.51 (m, 1H), 3.84 (m, 1H), 5.16 (m, 2H), 6.94 (m, 3H), 7.08-7.21 (m, 6H), 7.51 (m, 1H), 7.95 (m, 1H), 8.14 (s, 1H), 8.74 (br.s, 1H). ^{13}C NMR (150 MHz, CD$_3$OD): 20.1, 33.8, 40.7, 41.6, 42.1, 51.2, 52.1, 53.4, 62.8, 110.3, 119.1, 120.0, 124.1, 124.8, 126.6, 126.9, 127.0, 127.4, 128.3, 129.6, 131.0, 131.6, 133.9, 134.7, 136.1.

Preparation of **(6-chloro-3-(1-(4-chlorobenzyl)-4-(4-fluorophenyl)-1_H_-pyrazol-5-yl)-1_H_-indol-2-yl)((_S_)-3-((3-(dimethylamino)propyl)(methyl)amino)pyrrolidin-1-yl)methanone (24c, Method C):** The residue was purified by silica gel column chromatography (EtOAc/MeOH, 3/1; 0.5% Et$_3$N) to give the product as yellow solid (32 mg, 33% yield). HPLC/MS: t_R = 10.23 min; m/z = 647.4 [M+H]$^+$. HRMS: $C_{35}H_{38}N_6OCl_2F$, 647.2468 (calcd.), 647.2477 (found). ^{1}H NMR (600 MHz, CD$_3$OD, a mixture of rotamers): 1.32-1.35 (m, 2H), 1.55 (m, 1H), 1.86-1.95 (m, 4H), 2.01-2.14 (m, 6H), 2.22 (m, 1H), 2.36-2.46 (m, 2H), 3.15-3.22 (m, 3H), 3.32 (m, 1H), 5.26-5.28 (m, 2H), 6.80-6.87 (m, 3H), 7.07 (m, 1H), 7.15 (m, 1H), 7.28-7.33 (m, 5H), 7.99 (s, 1H). ^{13}C NMR (150 MHz, CD$_3$OD, a mixture of rotamers): 25.1, 25.3, 29.2, 30.8, 39.8, 39.9, 45.4, 45.5, 46.1, 48.1, 49.9, 50.7, 52.6, 54.7, 56.0, 56.1, 58.3, 58.6, 63.8, 64.8, 110.4, 112.6, 112.7, 116.4, 116.5, 116.7, 122.0, 122.1, 122.9, 123.4, 126.2, 126.5, 130.0, 130.1, 130.3, 130.6, 130.8, 131.0, 131.3, 131.6, 135.0, 135.1, 137.2, 137.4, 137.9, 144.0, 144.1, 153.6, 153.8, 153.9, 162.2, 163.9, 164.1, 164.2.

General procedure for the synthesis of compounds 25:

Preparation of **methyl 7-(6-chloro-3-(1-(4-chlorobenzyl)-4-phenyl-1_H_-pyrazol-5-yl)-1_H_-indole-2-carboxamido)heptanoate (25a, Method D):** The mixture of **22b** (43 mg, 0.09 mmol),

methyl 7-aminoheptanoate hydrochloride (23 mg, 0.1 mmol), N-methylmorpholine (NMM) (25 µL, 0.2 mmol) and HATU (2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate) (50 mg, 0.12 mmol) in 0.5 mL of DMF is heated for 1 h at 50 $^{\circ}$C. Ethylacetate is added and the organic layer is washed with brine, dried and evaporated in vacuo. The residue was purified by silica gel chromatography (EtOAc/hexanes, 1/1) to give the product as yellow solid (20 mg, 36% yield). HRMS: $C_{33}H_{32}N_4O_3Cl_2Na$, 625.1749 (calcd.), 625.1729 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.98 (m, 2H), 1.11-1.21 (m, 4H), 1.52-1.55 (m, 2H), 2.27-2.29 (m, 2H), 2.93-3.15 (2H, m), 3.69 (s, 3H), 4.94 (1H, ABd, J = 15.0 Hz), 5.14 (1H, ABd, J = 15.0 Hz), 5.72 (1H, m), 6.87 (m, 2H), 7.13-7.20 (8H, m), 7.59 (s, 1H), 8.03 (s, 1H), 10.88 (br.s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 8.6, 24.7, 26.3, 28.7, 28.8, 33.9, 39.4, 45.8, 51.5, 53.4, 104.1, 112.7, 121.3, 122.8, 123.6, 126.1, 126.5, 127.1, 128.7, 128.9, 129.3, 129.9, 130.5, 131.3, 131.4, 134.0, 134.5, 135.9, 138.6, 160.3, 174.1.

Preparation of **methyl 7-(6-chloro-3-(1-(4-chlorobenzyl)-4-(4-fluorophenyl)-1H-pyrazol-5-yl)-1H-indole-2-carboxamido)heptanoate (25b, Method D):** The residue was purified by silica gel chromatography (EtOAc/hexanes, 1/3) to give the product as yellow oil (37 mg, 33% yield). HPLC/MS: t_R = 13.07 min; m/z = 621.3 [M+H]$^+$. HRMS: $C_{33}H_{31}N_4O_3Cl_2FNa$, 643.1655 (calcd.), 643.1614 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.99-1.02 (m, 2H), 1.13-1.24 (m, 4H), 1.54-1.57 (m, 2H), 2.29-2.31 (m, 2H), 2.95-3.17 (2H, m), 3.71 (s, 3H), 4.95 (1H, ABd, J = 15.0 Hz), 5.16 (1H, ABd, J = 15.0 Hz), 5.63 (1H, m), 6.87-6.90 (m, 4H), 7.14-7.19 (m, 6H), 7.61 (s, 1H), 8.00 (s, 1H), 10.65 (br.s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 24.7, 26.3, 28.7, 28.8, 33.9, 38.6, 39.4, 51.6, 53.5, 103.8, 112.6, 115.9, 116.0, 121.2, 122.7, 122.9, 126.5, 127.7, 127.8, 128.7, 129.3, 129.9, 130.3, 131.5, 134.1, 134.4, 135.8, 138.4, 160.1, 174.1.

Preparation of **methyl 6-(6-chloro-3-(1-(4-chlorobenzyl)-4-(4-fluorophenyl)-1H-pyrazol-5-yl)-1H-indole-2-carboxamido)hexanoate (25c, Method D):** The residue was purified by silica gel chromatography (EtOAc/hexanes, 1/1) to give the product as yellow oil (17 mg, 38% yield). HPLC/MS: t_R = 12.73 min; m/z = 607.2 [M+H]$^+$. HRMS: $C_{32}H_{29}Cl_2FN_4O_3Na$, 629.1498 (calcd.), 629.1500 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.01-1.05 (m, 2H), 1.14-1.20 (m, 2H), 1.51-1.55 (m, 2H), 2.24-2.26 (m, 2H), 2.96-3.18 (m, 2H), 3.71 (s, 3H), 4.94 (1H, ABd, J = 15.0 Hz), 5.16 (1H, ABd, J = 15.0 Hz), 5.67 (m, 1H), 6.87-6.90 (m, 4H), 7.14-7.19 (m, 6H), 7.61 (s, 1H), 8.01 (s, 1H), 10.87 (br.s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 24.5, 26.1, 28.7, 33.8, 39.3, 51.7, 53.5, 103.8, 112.7, 115.9, 116.1, 121.2, 122.7, 122.9, 126.4, 127.49, 127.51, 127.7, 127.8, 128.7, 129.3, 129.8, 130.3, 131.5, 134.1, 134.4, 135.9, 138.4, 160.2, 161.0, 162.6, 173.9.

Preparation of **methyl 8-(6-chloro-3-(1-(4-chlorobenzyl)-4-(4-fluorophenyl)-1H-pyrazol-5-yl)-1H-indole-2-carboxamido)octanoate (25d, Method D):** The residue was purified by silica gel chromatography (EtOAc/hexanes, 1/3) to give the product as yellow oil (40 mg, 67% yield). HPLC/MS: t_R = 13.18 min; m/z = 635.3 [M+H]$^+$. HRMS: $C_{34}H_{33}Cl_2FN_4O_3Na$, 657.1811 (calcd.), 657.1833 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.99-1.01 (m, 2H), 1.13-1.27 (m, 6H), 1.59-1.63 (m, 2H), 2.31-2.33 (m, 2H), 2.98-3.17 (m, 2H), 3.70 (s, 3H), 4.94 (1H, ABd, J = 15.0 Hz), 5.15 (1H, ABd, J = 14.4 Hz), 5.67 (m, 1H), 6.86-6.88 (m, 4H), 7.14-7.19 (m, 6H), 7.61 (s, 1H), 8.00 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 24.8, 26.4, 28.8, 28.9, 34.0, 38.6, 39.5, 51.5, 53.4, 103.8, 112.8, 115.8, 116.0, 121.1, 122.7, 122.8, 126.4, 127.6, 127.7, 127.8, 128.7, 129.3, 129.9, 130.4, 131.4, 134.1, 134.5, 136.0, 138.4, 160.3, 161.0, 162.6, 174.2.

General procedure for the synthesis of compounds 26:

Preparation of **7-(6-chloro-3-(1-(4-chlorobenzyl)-4-phenyl-1H-pyrazol-5-yl)-1H-indole-2-carboxamido)heptanoic acid (26a, Method E):** The mixture of **25a** (0.025 mmol), THF (0.5

mL), water (0.5 mL), LiOH (10 mg) was stirring under RT for 2 days. The reaction mixture was acidified with 1M HCl (pH ~ 6). Then the mixture was extracted with DCM (30 mL x 3). The combined organic layer was dried over sodium sulfate, and evaporated. After removal of the solvents, 14 mg of yellow solid (95%) was obtained. HPLC/MS: t_R = 12.35 min; m/z = 589.2 [M+H]$^+$. HRMS: $C_{32}H_{30}N_4O_3Cl_2Na$, 611.1593 (calcd.), 611.1566 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.85-1.10 (m, 8H), 1.27-1.41 (m, 4H), 2.14 (m, 2H), 2.78-3.03 (m, 2H), 4.88 (1H, ABd, J = 14.4 Hz), 5.02 (1H, ABd, J = 13.8 Hz), 5.68 (s, 1H), 6.78-6.79 (m, 2H), 7.05-7.13 (m, 8H), 7.54 (s, 1H), 7.99 (s, 1H), 11.71 (br.s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 25.1, 27.8, 28.5, 29.7, 38.1, 53.1, 104.0, 112.7, 121.2, 122.6, 123.4, 126.1, 126.4, 127.0, 128.6, 128.9, 129.2, 129.8, 130.7, 131.1, 131.4, 133.9, 134.5, 136.1, 138.6, 160.6.

Preparation of **7-(6-chloro-3-(1-(4-chlorobenzyl)-4-(4-fluorophenyl)-1*H*-pyrazol-5-yl)-1*H*-indole-2-carboxamido)heptanoic acid (26b, Method E):** 25 mg of yellowish solid (95%) was obtained. HPLC/MS: t_R = 12.29 min; m/z = 607.1 [M+H]$^+$. HRMS: $C_{32}H_{29}N_4O_3Cl_2FNa$, 629.1498 (calcd.), 629.1552 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.92-1.28 (m, 8H), 1.57-1.60 (m, 2H), 2.35 (m, 2H), 2.92-3.14 (m, 2H), 4.94 (1H, ABd, J = 15.0 Hz), 5.15 (1H, ABd, J = 15.0 Hz), 5.66 (s, 1H), 6.86-6.89 (m, 4H), 7.14-7.33 (m, 6H), 7.62 (s, 1H), 8.01 (s, 1H), 10.95 (br.s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 24.6, 26.1, 28.5, 28.7, 33.8, 38.6, 39.3, 53.5, 103.9, 112.8, 115.9, 116.0, 121.1, 122.8, 122.9, 126.4, 127.7, 127.9, 128.7, 129.3, 129.7, 131.5, 134.1, 134.4, 135.9, 138.4, 160.3, 177.9.

Preparation of **6-(6-chloro-3-(1-(4-chlorobenzyl)-4-(4-fluorophenyl)-1*H*-pyrazol-5-yl)-1*H*-indole-2-carboxamido)hexanoic acid (26c, Method E):** 11 mg of yellowish solid (66%) was obtained. HPLC/MS: t_R = 11.98 min; m/z = 593.3 [M+H]$^+$. HRMS: $C_{31}H_{27}Cl_2FN_4O_3Na$, 615.1342 (calcd.), 615.1382 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.01-1.05 (m, 2H), 1.13-1.19

(m, 2H), 1.55-1.59 (m, 2H), 2.31 (m, 2H), 2.96-3.14 (m, 2H), 4.95 (1H, ABd, J = 15.0 Hz), 5.16 (1H, ABd, J = 15.0 Hz), 5.67 (m, 1H), 6.84-6.89 (m, 4H), 7.13-7.18 (m, 6H), 7.62 (s, 1H), 8.02 (s, 1H), 11.09 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 24.4, 25.9, 28.7, 29.7, 39.1, 53.5, 68.0, 103.9, 112.8, 115.9, 116.1, 121.1, 123.0, 126.3, 127.8, 128.7, 129.3, 129.6, 131.5, 134.1, 134.3, 136.0, 138.5, 160.4, 161.1, 162.7.

Preparation of **8-(6-chloro-3-(1-(4-chlorobenzyl)-4-(4-fluorophenyl)-1H-pyrazol-5-yl)-1H-indole-2-carboxamido)octanoic acid (26d, Method E):** 30 mg of yellowish solid (88%) was obtained. HPLC/MS: t_R = 12.35 min; m/z = 621.2 [M+H]$^+$. HRMS: C$_{33}$H$_{31}$Cl$_2$FN$_4$O$_3$Na, 643.1655 (calcd.), 643.1678 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.92-0.98 (m, 2H), 1.08-1.28 (m, 6H), 1.60-1.64 (m, 2H), 1.87-1.89 (m, 2H), 2.36-2.39 (m, 2H), 2.88-3.12 (m, 2H), 3.78 (m, 1H), 4.92 (1H, ABd, J = 15.0 Hz), 5.14 (1H, ABd, J = 15.0 Hz), 5.67 (m, 1H), 6.84-6.88 (m, 4H), 7.12-7.17 (m, 6H), 7.61 (s, 1H), 8.00 (s, 1H), 11.19 (br.s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 24.8, 25.6, 26.0, 28.4, 28.6, 38.6, 39.3, 53.4, 68.0, 103.9, 112.8, 115.9, 116.0, 121.1, 122.8, 122.9, 126.3, 127.4, 127.7, 127.8, 128.7, 129.3, 129.7, 130.5, 131.5, 134.1, 134.4, 136.0, 138.4, 160.4, 161.0, 162.7.

General procedure for the synthesis of compounds 27:

Preparation of **6-chloro-3-(1-(4-chlorobenzyl)-4-phenyl-1H-pyrazol-5-yl)-N-(7-(hydroxyl amino)-7-oxoheptyl)-1H-indole-2-carboxamide (27a, Method F):** The mixture of **26a** (0.025 mmol, 15 mg), hydroxylamine hydrochloride (0.06 mmol, 4.2 mg) and CDI (0.06 mmol, 9.72 mg) in 0.5 mL of THF was stirring under RT for 1 hour. The residue was purified by silica gel chromatography (EtOAc/hexanes, 2/1) to give the product as yellow solid (5 mg, 33% yield). HRMS: C$_{32}$H$_{31}$N$_5$O$_3$Cl$_2$Na, 626.1702 (calcd.), 626.1694 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.88-1.51 (m, 8H), 2.04-2.27 (m, 2H), 3.01-3.05 (m, 2H), 4.94 (1H, ABd, J = 16.2 Hz), 5.08

(1H, ABd, J = 15.0 Hz), 5.73 (s, 1H), 6.81-6.84 (m, 1H), 7.14 (m, 8H), 7.72 (m, 3H), 8.01 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 14.1, 22.7, 28.5, 29.4, 29.7, 31.9, 53.3, 103.9, 112.6, 121.3, 122.8, 123.7, 126.2, 126.4, 127.2, 128.7, 129.0, 129.3, 130.0, 130.8, 131.4, 134.0, 134.4, 138.6.

Preparation of **6-chloro-3-(1-(4-chlorobenzyl)-4-(4-fluorophenyl)-1***H***-pyrazol-5-yl)-N-(7-(hydroxyamino)-7-oxoheptyl)-1***H***-indole-2-carboxamide (27b, Method F):** The residue was purified by silica gel chromatography (EtOAc/hexanes, 1/1) to give the product as yellowish solid (10 mg, 49% yield). HRMS: C$_{32}$H$_{30}$N$_5$O$_3$Cl$_2$FNa, 644.1607 (calcd.), 644.1568 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.89-1.46 (m, 8H), 2.00 (m, 2H), 2.83-2.86 (m, 2H), 3.05-3.10 (m, 2H), 4.92-5.03 (m, 2H), 5.69 (s, 1H), 6.80-6.88 (m, 4H), 7.08-7.16 (m, 6H), 7.58 (s, 1H), 7.96 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 14.1, 22.7, 28.5, 29.4, 29.7, 31.9, 33.9, 36.5, 53.2, 56.0, 103.9, 115.8, 115.9, 121.1, 122.6, 123.0, 126.3, 127.8, 128.6, 128.7, 129.3, 130.7, 133.9, 134.5, 138.5, 160.6, 160.9.

Preparation of **6-chloro-3-(1-(4-chlorobenzyl)-4-(4-fluorophenyl)-1***H***-pyrazol-5-yl)-N-(6-(hydroxyamino)-6-oxohexyl)-1***H***-indole-2-carboxamide (27c, Method F):** The residue was purified by silica gel chromatography (EtOAc) to give the product as yellowish solid (11 mg, 54% yield). HPLC/MS: t_R = 11.98 min; m/z = 608.2 [M+H]$^+$. HRMS: C$_{31}$H$_{27}$Cl$_2$FN$_5$O$_3$, 606.1475 (calcd.), 606.1522 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.99-1.02 (m, 4H), 1.20-1.25 (m, 2H), 2.00-2.07 (m, 2H), 2.70-3.02 (m, 2H), 4.88 (1H, ABd, J = 13.8 Hz), 4.97 (1H, ABd, J = 13.8 Hz), 5.70 (m, 1H), 6.68-6.76 (m, 4H), 6.99-7.06 (m, 6H), 7.50 (s, 1H), 7.93 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 19.2, 22.7, 25.5, 28.8, 29.7, 53.1, 103.8, 112.7, 115.6, 115.8, 121.1, 122.4, 122.6, 126.3, 127.8, 128.6, 129.2, 129.8, 131.2, 133.8, 134.5, 136.3, 138.5, 160.8.

Preparation of **6-chloro-3-(1-(4-chlorobenzyl)-4-(4-fluorophenyl)-1***H***-pyrazol-5-yl)-N-(8-(hydroxyamino)-8-oxooctyl)-1***H***-indole-2-carboxamide (27d, Method F):** The residue was

purified by silica gel chromatography (EtOAc/hexanes, 1/1) to give the product as yellowish solid (16 mg, 52% yield). HPLC/MS: t_R = 12.31 min; m/z = 621.3 [M+H]$^+$. HRMS: $C_{33}H_{32}Cl_2FN_5O_3Na$, 658.1764 (calcd.), 658.1772 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.81-1.44 (m, 8H), 2.02-2.14 (m, 2H), 2.83-2.86 (m, 2H), 2.80-3.03 (m, 2H), 4.89-5.07 (m, 2H), 5.94 (s, 1H), 6.74-6.79 (m, 3H), 7.03-7.18 (m, 8H), 7.53 (s, 1H), 7.94 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 25.9, 26.0, 28.5, 28.8, 29.1, 29.7, 39.1, 39.2, 53.2, 104.2, 112.7, 115.8, 121.1, 122.4, 126.3, 127.8, 128.59, 128.61, 129.18, 129.23, 130.1, 131.1, 133.8, 134.6, 136.3, 138.3, 160.5, 160.8.

3.0   PROTEIN-PROTEIN INTERACTIONS DIRECTED LIBRARIES

3.1   INTRODUCTION

Designing small molecules that inhibit PPIs is a challenge area in the drug discovery arena.[15] It is widely accepted that this therapeutic class is difficult to tackle using traditional approaches, because the topology of the binding sites is different to more familiar protein classes, such as enzymes and GPCRs. HTS campaigns have so far been typified by the disappointingly low 'hit rate' of drugs targeting PPIs.[51] One of the most important obstacles to hit identification is that current chemical libraries do not fit well with the diversity of chemotypes present in protein-protein interaction inhibitors.[175] There is an emerging need to develop more efficient strategies for the collection of small molecules selectively targeting PPIs.

There is growing structural information on PPIs exemplified by PDB and the validated binding sites revealed by co-crystals of PPIs. It has been known that amino acids in PPI interfaces are not equally distributed; however, certain amino acids and clusters thereof are highly predominant.[176-180] In many cases, specific amino acid side chains of the donor protein are found deeply buried in the acceptor protein. These "anchor" motifs often play a critical role in PPIs by targeting relatively stable surface pockets on the receptor. Lacking biochemical mutational data, online tools are available to search the PDB for anchors,[62] revealing thousands of potential druggable PPIs that are "biased" to the known chemistry of these key residues. PDB-

wide statistics showed that aromatic (tryptophan, phenylalanine, tyrosine), hydrophobic (leucine, methionine) and hydrophilic (arginine, glutamine) side chains are the most enriched class of anchors among all interface residues in PPIs (**Figure 25**).
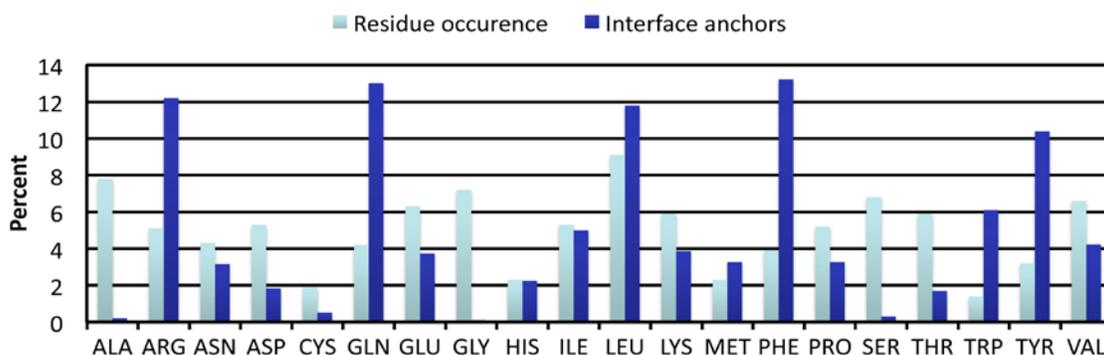
Figure 25. The distribution of the most deeply buried anchor (blue) with at least one anchor residue (ΔSASA > 80 Å^2 and >70% of SASA is buried), compared with the relative frequency of each residue in proteins.[151]

We hypothesize that libraries based around PPI specific motifs, rather than on the scaffold concept, will uncover a chemical space specifically directed to PPIs and provide useful, previously unexplored chemicals that are highly relevant to biological activity. These small molecules are preferably targeting a large subset of PPIs where dimension, shape and physicochemical nature of the interface implies "druggable" binding sites.

Based on these observations, we focus on the design and production of diverse, PPI-directed small molecule pilot libraries. MCR chemistry is an efficient "one-step, one pot" class of reactions that yield highly complex, drug-like and screening-ready products. Although not common in existing compound libraries, MCR compounds are well represented among known small-molecule PPI inhibitors.[27, 181, 182] More importantly, MCR derived peptidomimetic chemotypes allow us to design compound libraries that include chemical mimics of key amino acids important for molecular recognition.[183] Virtual libraries are created from an expanded set

of 23 MCR chemistries and starting materials that are curated for affordability, diversity, and synthesizability (**Appendix E**). To date, we have created larger and random anchor-oriented libraries for phenylalanine, tyrosine, tryptophan, and leusine (or valine) anchors, which are provided at http://anchorquery.ccbb.pitt.edu (**Figure 26**). About 100 conformations are generated with OpenEye Omega (http://www.eyesopen.com/omega), resulting in roughly 6×10^8 conformations per Anchor-based MCR library.

Anchor Biased Library
Compounds (21,628,988)

Reaction Class	Tryptophan	Tyrosine	Phenylalanine	Leucine/Valine
beta-Lactam	183,389	308,105	280,520	363,293
DKP	351,893	354,685	237,132	240,475
Doebner	48,185	42,888	72,291	16,770
Gewald	71,973	13,183	28,982	27,763
Groebke	113,329	73,200	162,103	122,529
Hydantoine	254,888	252,205	250,594	254,725
Imidazole	161,042	161,100	216,090	161,865
Isoquinoline	627,358	807,032	1,061,180	937,010
Orru	610,388	369,778	530,751	429,718
Orru+Amidation	300,206	399,364	399,863	400,502
PZQ	313,098	8,436	35,292	16,723
Reductive_Amination	208,314	204,864	252,633	262,708
Schollkopf	6,564	4,180	16,326	10,669
Schollkopf_amidation	250,826	250,068	249,952	250,993
Sulfonamide	101,137	100,340	150,595	153,101
Tetrazole	226,459	230,945	224,584	235,957
Thiazole	230,423	232,921	226,423	232,360
Thienodiazepine	172,571	124,177	296,357	296,937
U4C5Cr	251,138	241,488	216,856	231,575
UDC	234,629	244,126	237,526	239,734
Ugi_4_component	235,768	244,347	240,163	239,016
van_Leusen	152,723	151,871	150,845	154,923
Zhu	213,200	224,534	226,053	223,193
Total	**5,319,501**	**5,043,837**	**5,763,111**	**5,502,539**

Figure 26. Anchor biased virtual libraries (http://anchorquery.ccbb.pitt.edu/reactions/)

The diversity analysis of 16.8 million anchor-biased compounds and the 17.5 million compounds of the ZINC database[184] confirms that these MCR compounds encompass an untapped region of chemical space. The diversity space is visualized by plotting the top two principal components of the OpenBabel FP2 (http://openbabel.org) fingerprints of 200,000 compounds randomly selected equally from the 17.5 million compounds of the entire ZINC

database and the 16.8 million compounds comprising the publicly available phenylalanine, tyrosine, and tryptophan biased AnchorQuery libraries. PPI-biased AnchorQuery compounds are focused on untapped region of chemical space which is departure from historical targets, such as kinase inhibitors, but highly amenable to PPI targets, such as the p53/MDM2 interaction (**Figure 27**). A selection of kinase inhibitors, including inhibitors containing a tryptophan analog, falls squarely in the space covered by ZINC, while a selection of p53/MDM2 inhibitors, including inhibitors without a tryptophan analog, is located in the space covered by the AnchorQuery libraries.
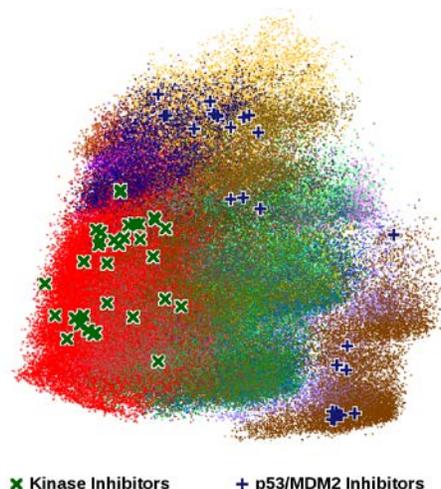
Figure 27. A representation of the chemical diversity of anchor-biased virtual libraries (shown in different colors) relative to the ZINC database (shown in red).[151] The diversity space is visualized by plotting the top two principal components of the OpenBabel FP2 (http://openbabel.org) fingerprints of 200,000 compounds randomly selected from the 17.5-and-16 million compounds of ZINC and anchor-biased database, respectively.

Libraries of amino acid motifs prevalent in PPI interfaces should have a much higher chance of yielding hits than the average, unbiased libraries. Therefore, we aim to generate PPI-directed small molecule libraries based on the validated anchor concept and commonly occurring amino acids in the PPI interfaces. Small molecules with these fragments that can mimic "anchor"

will be invaluable as inhibitors for critical PPIs, consequently providing vast opportunities for drug discovery for otherwise "undruggable" targets. This approach allows for the rapid design and synthesis of a library of compounds tailored to the exact interface of specific PPIs, facilitating screening for lead hits with known mechanism of actions.

3.2   DIVERSITY-ORIENTED SYNTHESIS VIA MULTICOMPONENT REACTIONS

Isocyanide-based multicomponent reaction (IMCR) chemistry is a truly efficient and versatile technique to synthesize diverse compounds, which are complementary to current screening libraries. The examples illustrated in **Chapter 3.2.1** have shown that drug-like compounds derived from MCRs are useful to target PPIs. Furthermore, **Chapter 3.2.2** explored the application of IMCR for the diversity-oritented synthesis of novel thiophene scaffolds, which access unexplored chemical space for new biological targets, such as PPIs.

3.2.1   Synthesis of drug-like compounds via MCRs

Diversity-oriented synthesis (DOS) of small molecules is an emerging field to identify new ligands for a variety of targets through libraries design.[185-187] So far, the efficient chemical synthesis of structurally and functionally diverse compounds still remains challenge.[188-190] MCR is generally defined as a reaction where more than two staring materials react to form a product, incorporating essentially all of the atoms of the educts. The MCR approach offers a straightforward route to generate complexity and diversity in a single operation. Many MCRs show advantages in atomic economy, environmental friendness, simplified steps, and efficient

use of resources.[191] Thus, MCR chemistry was employed as a powerful and efficient tool for the synthesis of drug-like compounds in a simple and economic manner.

a) p53/Mdm2 inhibitor

Piperazine is a chemical motif that consists of a six-membered ring containing two opposing nitrogen atoms. 73 Drug entries of piperazine derivatives are deposited in the Drug Bank (**Figure 28**). Isocyanide-based multicomponent reactions (IMCRs) allow for the convergent and efficient access to not less than 35 different piperazine derived scaffolds. [127, 192] Due to the boarderline nature of piperazine derivatives between small molecules and peptides they can be used as a logic pathway for the process of depeptisation: peptide => peptidomimetic => small molecule drug.
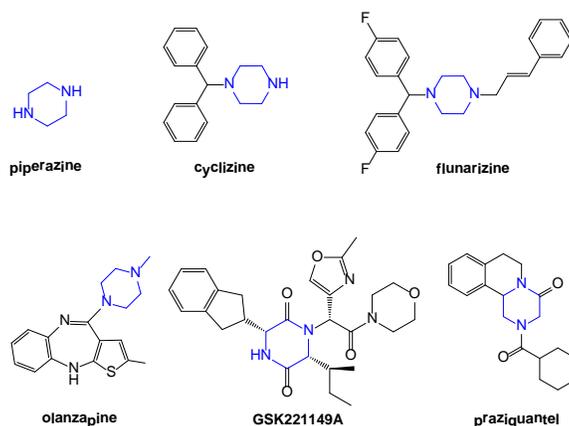
Figure 28. Representative structures of marketed and experimental drug entities containing piperazine scaffolds.

A 2,5-diketopiperazine compound **35** was synthesized via the reaction of an isocyanide **28**, an aromatic aldehyde **29**, a primary amine **30**, and chloroacetic acid **33**, followed by subsequent ring closure involving a nucleophilic substitution reaction (**Scheme 10**).[193] Compared with compound **32** (Ki = 37 µM), compound **35** (Ki = 36 µM) has shown similar activity against Mdm2 measured by FP assay. Based on the piperazine skeleton's favorable drug properties and evidenced by the scores of piperazine derivatives in notable successful drugs, this result

underscores its wide use in drug discovery as non-peptidic scaffolds for designing α-helix mimetics.
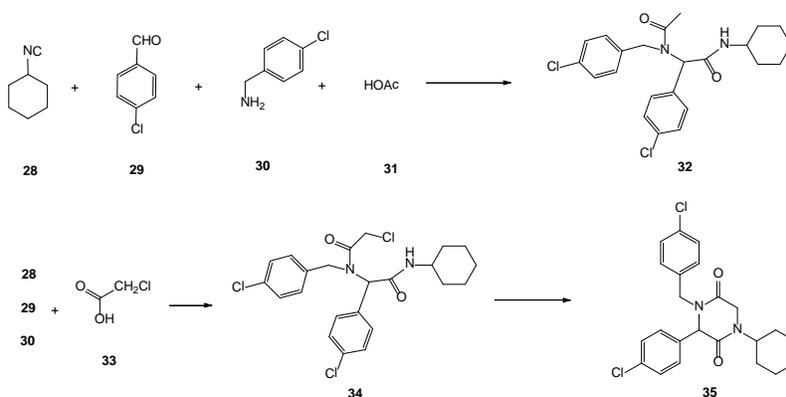
Scheme 10. Synthesis of 1,4-piperazine

b) HIV-1 gp41 inhibitor

The ectodomain of HIV-1 gp41 mediates the fusion of viral and host cellular membranes, which was further identified as a tractable drug target.[194] The peptide-based drug Enfuvirtide is precedent that inhibitors of this fusion activity may act as anti-HIV agents.[195] Upon gp120 binding to CD4 and a coreceptor (CCR5 or CXCR4), gp41 changes its conformation by forming N-helix trimer between N-heptad repeats (NHRs) and then six-helix bundle between the N-trimer and the C-heptad repeats (CHRs), as shown in **Figure 29a**.[196] Small molecule inhibitors targeting gp41 are being pursured in order to increase bioavailability and reduce the cost of production.[197]
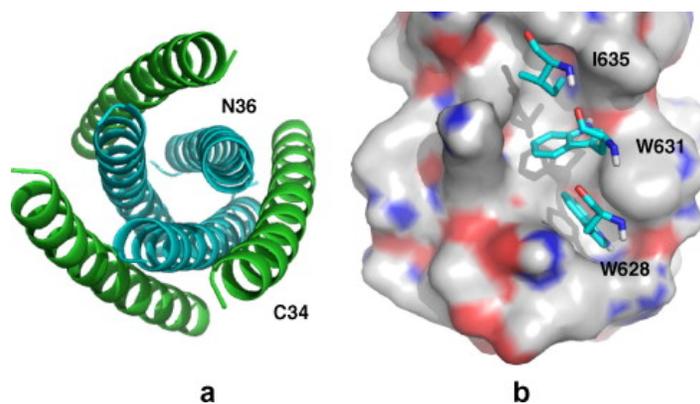
Evidence suggests that the cavity formed by the inner core trimer, which is occupied by C-peptide residues Trp628, Trp631, and Ile635, plays a crucial role in the stability of the hexamer (**Figure 29b**).[196] Small molecules which bind with high affinity into this pocket would inhibit six-helix bundle formation, thus exhibit an antiviral effect. Inspired by the successful examples of Ugi derived p53-Mdm2 inhibitors, two small molecules **39a-b** were designed to mimic the peptide α-helix, in which the hot spots consist of two Trp and iso-leucine. Therefore, the bis-indole scaffold was assembled by the Ugi-4CR, and followed by the cleavage of protection group (**Scheme 11**). These compounds were evaluated by collarbortors in Center for Vaccine Research.
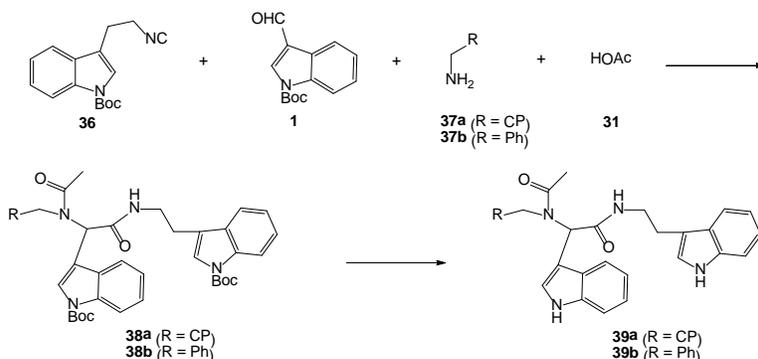
Scheme 11. Synthesis of anchor biased compounds

c) Capsid assembly inhibitor

Capsid (CA) assembly and disassembly are important processes in the HIV-1 life cycle,[198] thus ligands capable of interfering with these processes mediated by the CA protein are promising anti-retroviral drug candidates.[199] Amantadine is the first adamantane derivative used

as as an antiviral drug against various strains of flu and also to treat Parkinson's disease. Polymeric adamantane analogues were later developed as antiviral agents against HIV-1.[200] Virtual libraries assembled via efficient MCR using an adamantyl fragment were screened against crystal structure of full-length CA protein.[198] Compound **42** was synthesized by Passerini three-component reaction (P-3CR) with adamantine-carboxylic acid **40**, and cyclized to yield a highly substituted drug-like oxazole compound **43** (**Scheme 12**).[201] Compound **43** binds to native CA with micromolar affinity as measured by surface plasmon resonance (SPR), and has shown promising activity in a viral growth inhibition assay.[198]
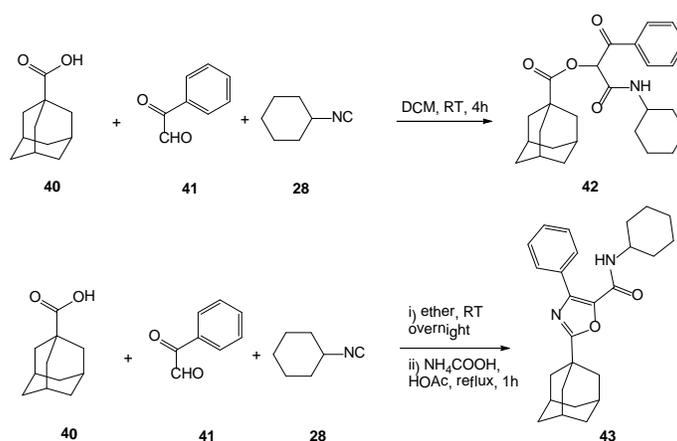
Scheme 12. Synthesis of HIV-1 capsid assembly inhibitor

d) p53/Mdm4 inhibitor

MCRs can address the requirements for efficient high-throughput synthesis of diverse compounds or drug candidates in a cost- and time-effective manner.[202] Armstrong and coworkers generated a 96-member library (12 acids, 8 aldehydes, 1 amine, and 1 isocyanide were the starting materials) in a 96-well microliter plate with a distribution of one product per well.[203] **Figure 30** illustrates structurally diverse 36 products distributed as one compound per well in a microliter plate, which employed 3 carboxylic acids, 3 aldehydes, 4 isocyanides, and 1 amine for

the Ugi reaction. Hulme and coworkers later developed low cost automation needs for plate based production and purification protocol using one step IMCR.[105]
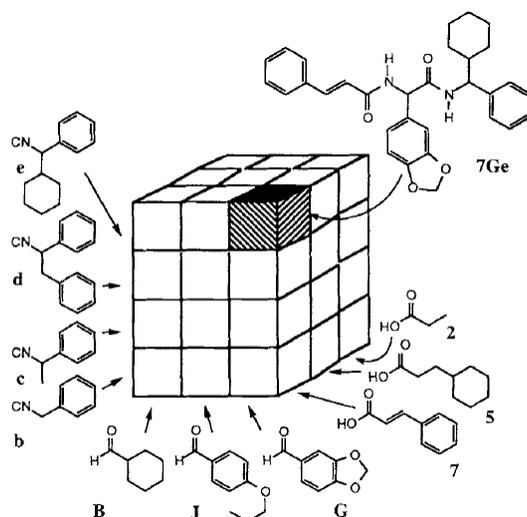
Figure 30. Chemical inputs and a selected product of the Ugi-4CR.[203] (Copyright permission submitted to John Wiley and Sons)

Thus, we generated a 96-member library of peptidomemetic small molecules via Ugi-4CR (**Scheme 13**). Each compound contains indole fragment to mimic Trp "anchor", which is commonly present in protein-protein interaction interface. **Figure 31** illustrates the structure of amine and isocyanide inputs, as well as the experimental setting in a 96-well microliter plate. The products were collected by a 96-well filter plate, and washed with ether to remove unreacted starting materials. The collected samples were dissolved as a 10 mM stock solution in DMSO for the screening purpose.
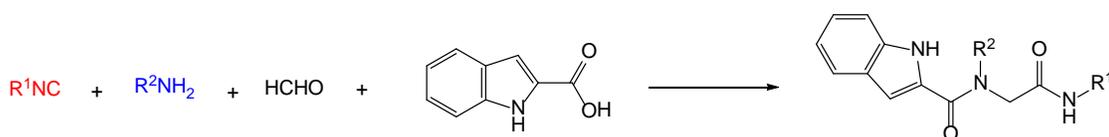
Scheme 13. Ugi-4CR for high throughput synthesis

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Figure 31. High-throughput synthesis of "anchor" biased compound library via Ugi-4CR. A: amine input; B: isocyanide input; C: experimental setting.
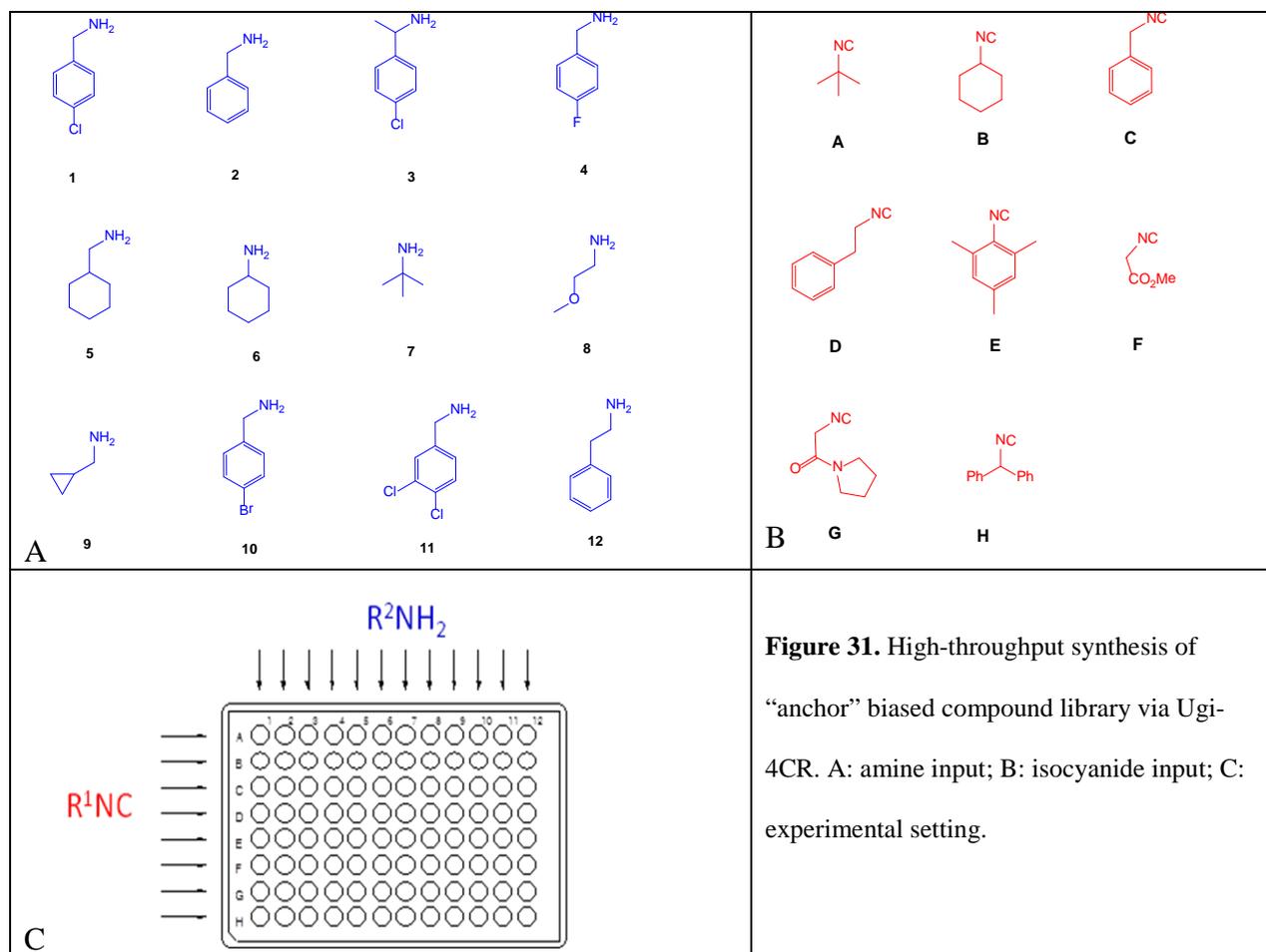
Compound B1 was identified as p53/Mdm4 inhibitor via FP assay (Ki = 5.5 µM), as shown in **Figure 32**. As far as it concerns small molecules, the availability of dual and/or selective Mdm4 inhibitors is still poor compared to Mdm2 inhibitors,[57, 204] with very few small molecules being reported as selective modulator of Mdm4.[205, 206] Although the p53-binding sites within the Mdm4 and Mdm2 proteins are closely related, known Mdm2 small-molecule inhibitors have been shown experimentally not to bind to its homolog Mdm4.[207] This hit compound may provide a new avenue for the identification of potential selective inhibitors of p53-Mdm4 interaction.

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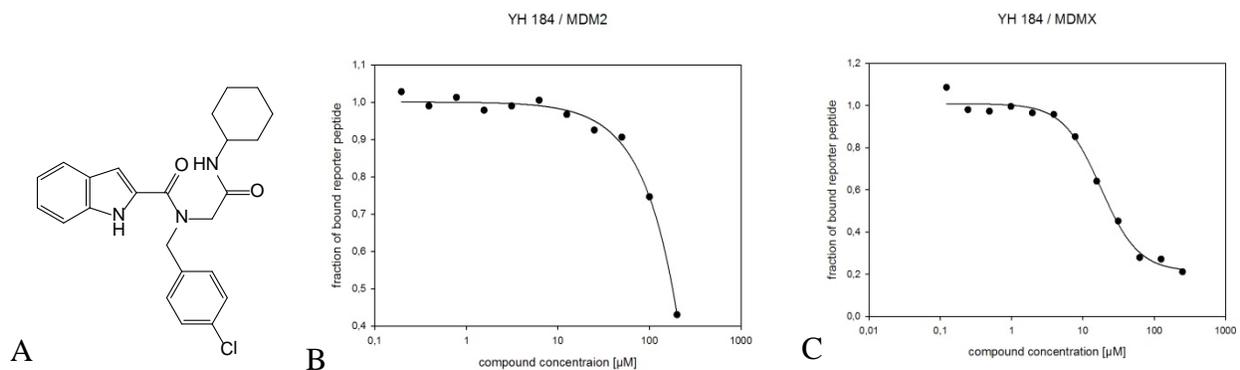
Figure 32. Hit compound as p53/Mdm4 inhibitor. A: Structure of B1; B: Ki = 54 µM (Mdm2) C: Ki = 5.5 µM (Mdm4)

Materials and Methods

Preparation of **2-(*N*-(4-chlorobenzyl)acetamido)-2-(4-chlorophenyl)-*N*-cyclohexylacetamide (32):** The mixture of 4-chloro-benzyladehyde (0.2 mmol, 28.0 mg), 4-chloro-benzylamine (0.2 mmol, 24.4 µL), cyclohexyl isocyanide (0.2 mmol, 24.9 µL), acetic acid (0.2 mmol, 11.5 µL) in 0.5 mL of methanol was stirring under RT for 2 days. The product was purified by chromatography on silica gel (petroleum ether/ethyl acetate, 2:1) as white solid (70 mg, yield: 81%). HPLC/MS: t_R = 11.75 min; m/z = 433.0 [M+H]$^+$. HRMS: $C_{23}H_{26}Cl_2N_2O_2$, 432.13713 (calcd.), 432.137631 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.09-1.11 (m, 3H), 1.28-1.34 (m, 2H), 1.56-1.65 (m, 3H), 1.85 (m, 2H), 2.04 (s, 3H), 3.74 (m, 1H), 4.51 (ABd, 1H, *J* = 17.4 Hz), 4.70 (ABd, 1H, *J* = 18.0 Hz), 6.00 (s, 1H), 6.08 (s, 1H), 6.93 (d, 2H, *J* = 8.4 Hz), 7.16 (d, 2H, *J* = 8.4 Hz), 7.21 (d, 2H, *J* = 8.4 Hz), 7.26 (d, 2H, *J* = 7.8 Hz). ^{13}C NMR (150 MHz, CDCl$_3$): 22.4, 24.7, 24.8, 25.4, 32.7, 48.6, 50.0, 61.3, 127.4, 128.6, 128.9, 130.9, 132.8, 133.8, 134.6, 136.0, 168.2, 172.6.

Preparation of **2-chloro-*N*-(4-chlorobenzyl)-*N*-(1-(4-chlorophenyl)-2-(cyclohexylamino)-2-oxoethyl)acetamide (34):** The mixture of 4-chloro-benzyladehyde (0.2 mmol, 28.0 mg), 4-

chloro-benzylamine (0.2 mmol, 24.4 μL), cyclohexyl isocyanide (0.2 mmol, 24.9 μL), acetic acid (0.2 mmol, 18.9 mg) in 0.5 mL of methanol was stirring under RT for 2 days. The product was purified by chromatography on silica gel (petroleum ether/ethyl acetate, 2:1) as white solid (53 mg, yield: 57%). HRMS: $C_{23}H_{25}Cl_3N_2O_2Na$, 489.0879 (calcd.), 489.0870 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.08-1.13 (m, 3H), 1.29-1.35 (m, 2H), 1.58-1.66 (m, 3H), 1.87 (m, 2H), 3.75 (m, 1H), 3.93 (ABd, 1H, J = 12.0 Hz), 4.01 (ABd, 1H, J = 12.6 Hz), 4.54 (ABd, 1H, J = 17.4 Hz), 4.75 (ABd, 1H, J = 17.4 Hz), 5.86 (s, 1H), 7.00 (m, 2H), 7.21 (m, 2H), 7.27 (m, 4H). ^{13}C NMR (150 MHz, CDCl$_3$): 24.7, 24.8, 25.4, 32.7, 41.9, 48.8, 49.5, 62.4, 127.4, 128.9, 129.2, 130.8, 133.0, 133.4, 135.0, 135.1, 167.5, 168.3.

Preparation of **4-(4-chlorobenzyl)-3-(4-chlorophenyl)-1-cyclohexylpiperazine-2,5-dione (35):** **34** was treated with a solution of KOH (0.2 mmol, 11.2 mg) in 0.5 mL of ethanol, and stirring under RT overnight. The product was purified by chromatography on silica gel (petroleum ether/ethyl acetate, 3:1) as white solid (40 mg, yield: 82%). HRMS: $C_{23}H_{24}Cl_2N_2O_2Na$, 453.1113 (calcd.), 453.1128 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.07-1.84 (m, 10H), 3.61 (ABd, 1H, J = 15.0 Hz), 3.94 (ABd, 1H, J = 17.4 Hz), 3.94 (ABd, 1H, J = 17.4 Hz), 4.23 (m, 1H), 4.86 (s, 1H), 5.40 (ABd, 1H, J = 14.4 Hz), 7.12 (d, 2H, J = 8.4 Hz), 7.23 (d, 2H, J = 8.4 Hz), 7.29 (m, 2H), 7.37 (m, 2H). ^{13}C NMR (150 MHz, CDCl$_3$): 25.2, 25.3, 25.4, 29.1, 29.4, 44.6, 46.9, 52.8, 62.8, 127.7, 129.2, 129.5, 129.9, 133.4, 133.5, 134.2, 134.9, 163.7, 165.0.

Preparation of *tert*-butyl 3-(2-(2-(1-(*tert*-butoxycarbonyl)-1*H*-indol-3-yl)-2-(*N*-(cyclopropyl methyl)acetamido)acetamido)ethyl)-1*H*-indole-1-carboxylate (38a): The mixture of *tert*-butyl 3-formyl-1*H*-indole-1-carboxylate (0.2 mmol, 49 mg), cyclopropylmethanamine (0.2 mmol, 17.1 μL), *tert*-butyl 3-(2-isocyanoethyl)-1*H*-indole-1-carboxylate (0.2 mmol, 0.2 mmol, 54 mg), acetic acid (0.2 mmol, 11.5 μL) in 0.5 mL of methanol was stirring under RT for 2 days. The

product was purified by chromatography on silica gel (petroleum ether/ethyl acetate, 3:1) as white solid (88.5 mg, yield: 70%). HPLC/MS: t_R = 13.14 min; m/z = 628.8 $[M+H]^+$. HRMS: $C_{36}H_{44}N_4NaO_6$, 651.3159 (calcd.), 651.3138 (found). ^{1}H NMR (600 MHz, CDCl$_3$): -0.27 (m, 1H), 0.06-0.09 (m, 2H), 0.30 (m, 1H), 0.58 (m, 1H), 1.67 (s, 9H), 1.69 (s, 9H), 2.21 (s, 3H), 2.93-2.97 (m, 2H), 3.12 (d, 1H, J = 6.0 Hz), 3.62-3.67 (m, 2H), 6.39 (s, 1H), 6.69 (s, 1H), 7.18-7.24 (m, 2H), 7.30-7.32 (m, 3H), 7.43 (s, 1H), 7.55 (m, 1H), 8.13-8.17 (m, 3H). ^{13}C NMR (150 MHz, CDCl$_3$): 4.4, 4.7, 10.9, 22.1, 25.0, 28.2, 29.3, 50.6, 53.2, 83.5, 84.1, 114.1, 115.29, 115.31, 117.5, 118.7, 118.9, 122.5, 122.9, 123.2, 124.5, 124.8, 127.1, 129.8, 130.3, 135.1, 135.5, 149.6, 169.9, 172.0.

Preparation of *N*-(2-(1*H*-indol-3-yl)ethyl)-2-(*N*-(cyclopropylmethyl)acetamido)-2-(1*H*-indol-3-yl)acetamide (39a): 38a (43 mg) was treated with a solution of K$_2$CO$_3$ (41.4 mg, 0.3 mmol) in MeOH-H$_2$O (3:1, 1 mL) stirring overnight under reflux. The product was purified by chromatography on silica gel (petroleum ether/ethyl acetate, 1:2) as yellowish solid (19 mg, yield: 67%). HRMS: $C_{26}H_{28}N_4NaO_2$, 451.2110 (calcd.), 451.2108 (found). ^{1}H NMR (600 MHz, CDCl$_3$): -0.39 (m, 1H), -0.02-0.05 (m, 2H), 0.27 (m, 1H), 0.56 (m, 1H), 2.19 (s, 3H), 2.93-2.95 (m, 2H), 3.10-3.13 (m, 2H), 3.56-3.63 (m, 2H), 6.33 (s, 1H), 6.39 (s, 1H), 6.88 (s, 1H), 7.05-7.22 (m, 4H), 7.32-7.54 (m, 5H), 8.34 (s, 1H), 8.69 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 4.1, 4.5, 10.9, 22.2, 25.0, 39.7, 51.0, 54.6, 109.6, 111.2, 111.4, 112.6, 118.5, 119.3, 120.1, 122.0, 122.4, 122.5, 125.9, 127.2, 135.8, 136.4, 170.5, 171.8.

Preparation of *tert*-butyl 3-(2-(2-(*N*-benzylacetamido)-2-(1-(*tert*-butoxycarbonyl)-1*H*-indol-3-yl)acetamido)ethyl)-1*H*-indole-1-carboxylate (38b): The mixture of *tert*-butyl 3-formyl-1*H*-indole-1-carboxylate (0.2 mmol, 49 mg), benzyl amine (0.2 mmol, 21.9 µL), *tert*-butyl 3-(2-isocyanoethyl)-1*H*-indole-1-carboxylate (0.2 mmol, 0.2 mmol, 54 mg), acetic acid (0.2 mmol,

11.5 µL) in 0.5 mL of methanol was stirring under RT for 2 days. The product was purified by chromatography on silica gel (petroleum ether/ethyl acetate, 3:1) as white solid (104 mg, yield: 78%). HPLC/MS: t_R = 13.16 min; m/z = 665.0 [M+H]$^+$. HRMS: $C_{39}H_{44}N_4NaO_6$, 687.3159 (calcd.), 687.3157 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.65 (s, 18H), 2.91-2.98 (m, 2H), 3.45 (s, 3H), 3.61-3.64 (m, 2H), 4.60-4.68 (m, 2H), 6.51 (s, 1H), 6.82-6.86 (m, 3H), 7.02-7.22 (m, 5H), 7.30-7.52 (m, 5H), 7.89 (s, 1H), 8.07-8.12 (m, 2H). ^{13}C NMR (150 MHz, CDCl$_3$): 22.5, 25.0, 28.15, 28.19, 39.5, 49.8, 50.5, 53.0, 83.5, 84.1, 113.9, 115.3, 117.6, 118.8, 118.9, 122.5, 123.0, 123.2, 124.5, 124.9, 125.9, 126.8, 127.4, 128.2, 129.6, 130.3, 135.1, 137.3, 149.3, 149.7, 169.3, 172.9.

Preparation of ***N*-(2-(1*H*-indol-3-yl)ethyl)-2-(*N*-benzylacetamido)-2-(1*H*-indol-3-yl)acetamide (39b): 38b** was treated with a solution of K$_2$CO$_3$ (70 mg, 0.5 mmol) in MeOH-H$_2$O (3:1, 1 mL) stirring overnight under reflux. The product was purified by chromatography on silica gel (petroleum ether/ethyl acetate, 1:2) as yellowish solid (40 mg, yield: 55%). HRMS: $C_{29}H_{28}N_4NaO_2$, 487.2110 (calcd.), 487.2139 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.99 (s, 3H), 2.88 (m, 2H), 3.47-3.62 (m, 2H), 4.52-4.58 (m, 2H), 6.24 (m, 1H), 6.39 (s, 1H), 6.75 (s, 1H), 6.87-6.88 (m, 2H), 7.03-7.24 (m, 6H), 7.30-7.55 (m, 6H), 8.44 (s, 1H), 8.82 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 22.5, 25.0, 39.8, 50.3, 50.7, 54.8, 109.1, 111.3, 111.5, 112.3, 118.57, 118.60, 119.2, 120.2, 121.9, 122.5, 126.1, 126.2, 126.8, 127.1, 127.2, 127.9, 128.3, 128.8, 135.8, 136.4, 137.6, 170.3, 172.8.

Preparation of **1-(cyclohexylcarbamoyl)-2-oxo-2-phenylethyl adamantine-1-carboxylate (42)**: The mixture of 1-adamantanecarboxylic acid (0.2 mmol, 36 mg), phenylglyoxal hydrate (0.2 mmol, 31.3 mg), cyclohexyl isocyanide (0.2 mmol, 24.9 µL), and 0.5 mL of DCM was stirring for 4 hours under room temperature. The product was isolated by silica gel chromatography

(hexanes/ethyl acetate, 5:1) as white solid (81 mg, 96%). HPLC/MS: t_R = 13.07 min; m/z = 424.2 [M+H]$^+$. HRMS: $C_{26}H_{34}NO_4$, 424.2488 (calcd.), 424.2505 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.19-1.38 (m, 5H), 1.59-2.08 (m, 20H), 3.76 (m, 1H), 6.20 (d, 1H, J = 7.8 Hz), 6.26 (s, 1H), 7.49-7.50 (m, 2H), 7.60 (m, 1H), 8.13-8.14 (m, 2H). ^{13}C NMR (150 MHz, CDCl$_3$): 24.5, 24.6, 25.4, 27.8, 32.6, 32.7, 36.3, 36.7, 40.6, 48.4, 75.8, 128.5, 129.7, 134.0, 134.5, 163.0, 175.3, 192.0.

Preparation of **2-(adamantan-1-yl)-*N*-cyclohexyl-4-phenyl-1,3-oxazole-5-carboxamide (43)**: The mixture of 1-adamantanecarboxylic acid (4 mmol, 0.72 g), phenylglyoxal hydrate (4 mmol, 0.63 g), cyclohexyl isocyanide (4 mmol, 498 µL), and 5 mL of ether was stirring overnight under room temperature. After the evaporation of the solvent, the white solid were treated with ammonium formate (76 mmol, 4.8 g), and acetic acid (10 mL). The mixture was heated under reflux for 1 hour, and then allowed to stand overnight. The product was isolated by simple filtration as yellow solid (1.47 g, 91%). HPLC/MS: t_R = 14.46 min; m/z = 405.1 [M+H]$^+$. HRMS: $C_{26}H_{33}N_2O_2$, 405.2542 (calcd.), 405.2534 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.21-1.46 (m, 5H), 1.66-2.14 (m, 20H), 3.97 (m, 1H), 6.07 (d, 1H, J = 7.8 Hz), 7.37-7.45 (m, 3H), 8.17-8.19 (m, 2H). ^{13}C NMR (150 MHz, CDCl$_3$): 24.9, 25.5, 27.9, 33.2, 35.8, 36.4, 40.3, 48.3, 128.1, 129.1, 129.3, 130.8, 138.1, 143.0, 157.3, 169.8.

High throughput synthesis of a 96-member library:

Add indole (200 µL, 1M methanol stock solution; 0.2 mmol), amine (200 µL, 1M methanol stock solution; 0.2 mmol), isocyanide (200 µL, 1M methanol stock solution; 0.2 mmol), formaldehyde (120 µL, 2M methanol stock solution; 0.24 mmol) into each well of 96 deep well plate with printed labeling (VWR D108839, 1.2 mL). The plated was sealed by aluminum foil sealing film and was µLtrasound for 1h, then stand overnight under RT. After the evaporation of

the solvent, the product was collected by filtration and washed by ether (AcroPrep[TM] 96 filter plate from PALL (0.2 um GHP, 1 mL well) was used, 96 deep well plate was used as the receiver).

Characterization of **N-(4-chlorobenzyl)-N-(2-(cyclohexylamino)-2-oxoethyl)-1H-indole-2-carboxamide (B1):** white solid, 20.6 mg; yield: 24%. HPLC/MS: t_R = 11.61 min; m/z = 424.2 [M+H]$^+$. HRMS: $C_{24}H_{26}N_3O_2ClNa$, 446.1611 (calcd.), 446.1634 (found). ^{1}H NMR (600 MHz, d^6-DMSO, a mixture of rotamers): 1.18-1.27 (m, 5H), 1.53-1.74 (m, 4H), 3.58 (m, 1H), 3.94 (m, 1H), 4.18 (m, 1H), 4.66 (m, 1H), 5.00 (m, 1H), 6.53 (m, 1H), 6.75 (m, 1H), 7.03 (m, 1H), 7.18 (m, 1H), 7.37-7.56 (m, 4H), 7.84 (m, 1H), 8.05 (m, 1H), 11.70 (m, 1H). ^{13}C NMR (150 MHz, d^6-DMSO, a mixture of rotamers): 24.4, 25.1, 32.2, 47.5, 49.7, 51.2, 54.9, 103.9, 111.99, 112.04, 119.8, 121.4, 123.5, 126.8, 128.3, 129.8, 131.8, 135.9, 136.3, 163.8, 166.9.

3.2.2   Discovery of novel aminothiophene scaffolds

Thiophene ring as a popular bioisostere of phenyl ring is becoming a more common component of potential therapeutic agents.[208] Therefore, the development of new thiophene scaffolds is desirable to increase the diversity of compound libraries. Gewald three-component reaction (Gewald-3CR) is a unique method using elemental sulfur to yield 2-aminothiophenes, which builds a platform for the synthesis of new thiophene scaffolds.[209, 210] The application of Gewald aminothiophene synthesis has already been rewarding for the pharmaceutical industry. Olanzepine is an atypical antipsychotic drug used in the treatment of schizophrenia and bipolar disorder since 1996.[211] This drug is manufactured by Eli Lilly in a very concise way, due to the formation of its thiophene ring by G-3CR in the first step (**Scheme 14**).[212] In search of novel and

efficient synthetic routes for the construction of thiophene scaffolds, 2-aminothiophene has been considered as a very appropriate precursor.[213]
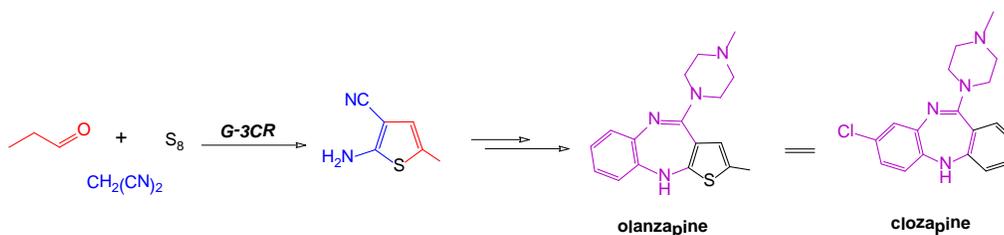
Scheme 14. Eli Lilly synthesis of olanzapine

In recent years, an increasing number of drug candidates based on 2-aminothiophene fragments has been developed.[214] Such thiophene scaffolds driven from Gewald reaction have been found with a variety of pharmacological activities (**Figure 33**). For example, AX20017 has been identified as a specific inhibitor of protein kinase G (IC_{50} = 0.39 μM).[215] GlaxoSmithKline developed a lead compound **44** as JNK kinase inhibitor (pIC_{50} = 6.4, JNK3).[216] Novo Nordisk discovered **45** as a selective inhibitor of protein tyrosine phosphatase 1B (PTP1B).[217] Thus, we are particularly interested in the development of new thiophene scaffolds starting from G-3CR.
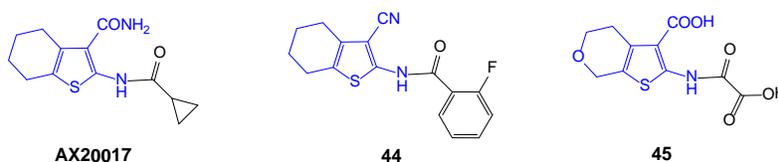
Figure 33. Drug candidates containing 2-aminothiophene scaffolds

However, the diversity of Gewald aminothiophene scaffolds is limited due to the available synthetic methods (*N*-acylation, *N*-alkylation, etc.). With the emergence of combinatorial chemistry and HTS for drug discovery applications, MCR provides a powerful tool for producing diverse arrays of compounds.[107] The development of new MCR approach is potential useful to explore diverse chemotypes used to probe biological targets, such as enzymes,

GPCRs and PPIs.[218] IMCRs are widely recognized in assembling complex pharmacologically important structures in a small number of steps with the additional point of diversity.[102] Although IMCRs could enlarge the chemical space of thiophene scaffolds, the applications of Gewald aminothiophenes for Ugi and Passerini reaction have been rarely reported so far. Our recent studies have successfully applied thiophene carboxylic acids **47** derived from Gewad products **46** as acid components for the synthesis of 1,4-thienodiazepine-2,5-diones (TDZs) **48** via Ugi-Deprotection-Cyclization (UDC) strategy (**Scheme 15**).[125, 219] Therefore, we intended to systematically investigate the reactivity of Gewald thiophene scaffolds for IMCRs.
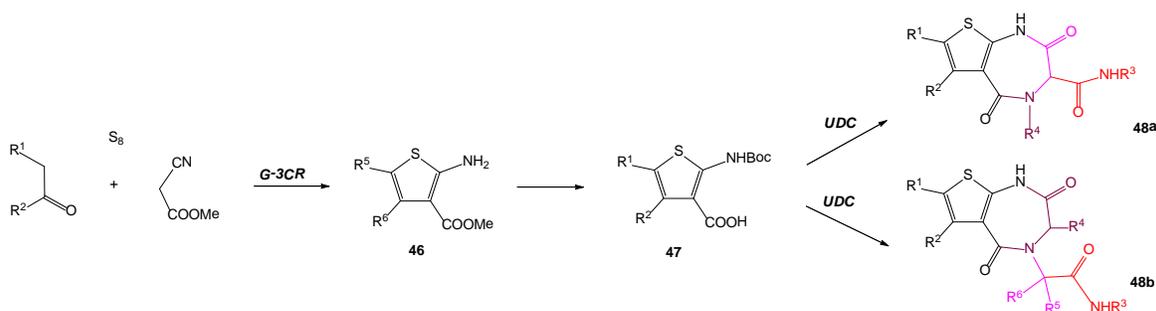
Scheme 15. Synthesis of 1,4-thienodiazepine-2,5-diones via UDC strategy

Dipeptidomimetic derivatives have garnered intense research interest for the development of lead structures, such as protease inhibitors.[220] Ugi-4CR is known to be one of the most versatile tool for the construction of dipeptide backbones.[102] The starting materials 2-aminothiophenes were prepared using the one-pot procedures of the Gewald method in multi-gram scales. We synthesized **46a** and **46b** via Gewald-3CR of cyclohexanone, sulfur and methyl cyanoacetate or malononitrile in the presence of diethylamine.[221] The products were precipitated and collected by filtration without further purification. The reactivity of Gewald 2-aminothiophenes was examined as amine components by U-4CR (**Scheme 16**). The dipeptide

derivatives of Gewald thiophene scaffold **49a** and **49b** were obtained by the Ugi reaction of aminothiophene **46a** or **46b**, with formaldehyde, acetic acid, and phenylethyl isocyanide.
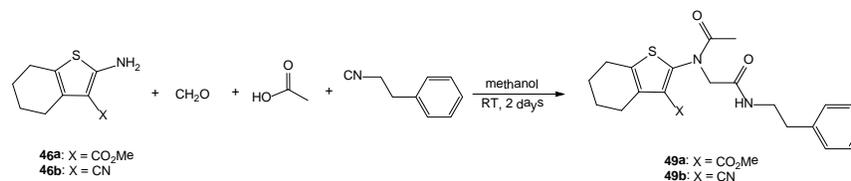
Scheme 16. Synthesis of dipeptide derivatives

Tetrazole compounds continuously emerge as lead structures for novel pharmaceuticals, such as inhibitors of HIV protease.[222] In order to enhance pharmacological properties of peptidomimetics, 1,5-disubstituted tetrazole ring is known to be an excellent mimic of a *cis*-amide bond.[223] The tetrazole derivatives of Gewald thiophene scaffold **50** were obtained by the reaction of aminothiophene **46a** or **46b**, *tert*-butyl isocyanide, isobutylaldehyde, and trimethylsilyl azide (**Scheme 17**). The real reactant hydrazoic acid was generated in situ by the reaction of trimethylsilyl azide with methanol.[193]
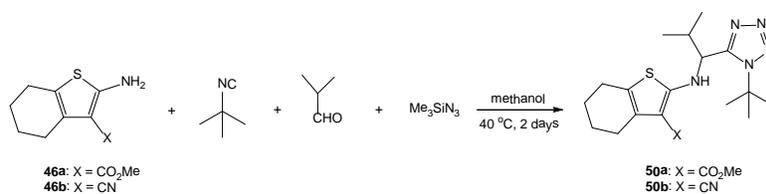
Scheme 17. Synthesis of tetrazole derivatives

Based on the above results, the conversion of **46b** is generally lower than **46a** due to the stronger electron withdrawing ability of the nitrile group. The amine functionality is deactivated due to electronic effects of the thiophene ring, which drastically affects the electron density of C-2.[224] However, most 2-aminothiophenes according to Gewald reactions have an electron withdrawing group (EWG) at C-3, which is introduced by an activated nitrile for the

Knoevenagel condensation with an oxo component. Therefore, the amino group is less reactive than aniline due to the adjacent thiophene ring and substitution at C-3.

α-Amino amide group is a frequently recurring motif in many lead compounds, such as inhibitors of dipeptidyl peptidase IV.[225] Therefore, we intended to transform 2-aminothiophenes as substrates for Ugi three-component reaction (U-3CR), which yields α-amino amide in the absence of an acid component. Gewald 2-aminothiophene **46c** was prepared by the treatment of **46b** with sulfuric acid.[226] The reactivity of **46c** was examined as an amine component by intramolecular U-3CR (**Scheme 18**).[227, 228] We speculated that this intramolecular process could overcome the reactivity issue of deactivated amine.
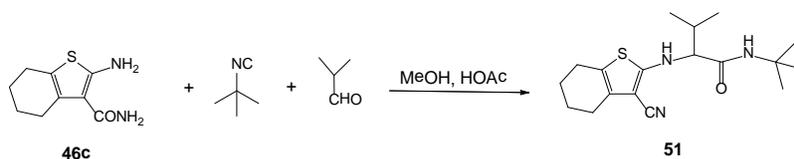
Scheme 18. Synthesis of α-amino amide derivative

The reactivity of Gewald 2-aminothiophene was examined as an isocyanide component by U-3CR (**Scheme 19**). The corresponding isocyanide derivative **53** was obtained by the two-step functional group transformations.[229] In the first step, the formamide intermediate **52** was obtained in 91% yield by the treatment of Gewald product **46a** with formic acid. In the second step, the formamide product **52** undergoes the dehydration process to give isocyanide derivative **53** in nearly quantitative conversion. The α-amino amide derivative of Gewald thiophene scaffold **54** can be synthesized by the catalytic U-3CR.[230] The reaction of the isocyanide derivative **53**, isobutylaldehyde, benzylamine, and the catalytic ammount of phenyl phosphinic acid gave the α-amino amide derivative **54**.
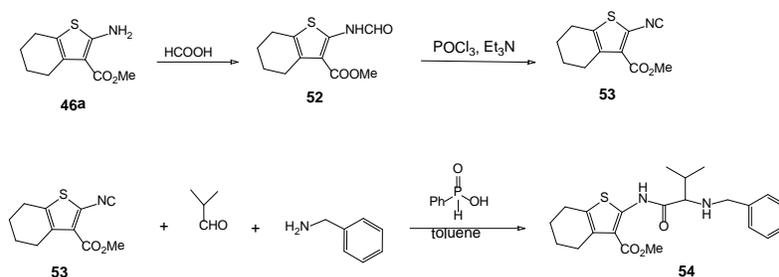
Scheme 19. Ugi three-component reaction

The synthesis of *α*-acyloxy amide derivatives has attracted much attention as these compounds provide access to new drug candidates, such as mandipropamid.[231] P-3CR offers direct access to α-acyloxy amides.[232] The Gewald thiophene scaffold **53** was employed to synthesize α-acyloxy amide derivative **55** by P-3CR (**Scheme 20**). However, the isocyanide functionality is also deactivated due to the adjacent thiophene ring and an electron withdrawing substituent at C-3.
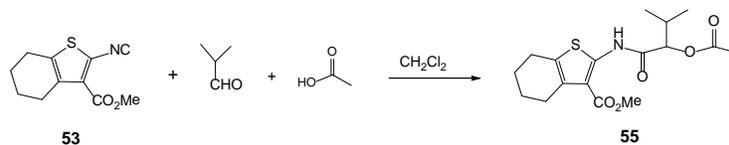
Scheme 20. Synthesis of *α*-acyloxy amide derivative

We have shown that thiophene carboxylic acids **47** derived from Gewad products **46** were successfully applied for U-4CR.[233] Thus, **47a** and **47b** were employed as acid components for P-3CR (**Scheme 21**). Obviously, the reactivity of carboxylic acid functionality is unaffected by thiophene scaffolds. *α*-Acyloxy amide derivatives **56** were obtained by the P-3CR and subsequent deprotection of Boc under TFA. Moreover, the intramolecular cyclization was not observed by the treatment of **56** with the catalytic amount of TBD.
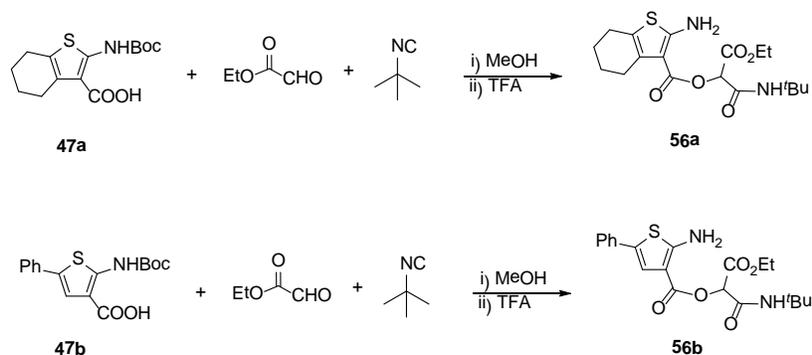
Scheme 21. Synthesis of α-acyloxy amide derivatives

2-Aminothiophene **57** was synthesized by the Gewald reaction of 1,4-dithiane-2,5-diol and methyl cyanoacetate (**Scheme 22**).[234] The dipeptide derivative **58** was obtained by the Ugi reaction of aminothiophene **57**, formaldehyde, acetic acid, and phenylethyl isocyanide. Obviously, 2-aminothiophene could act as an isostere of phenyl ring according to structural similarity and reaction type. Based on the synthetic methodology we have investigated, **47c** was employed for the synthesis of dipeptide derivative **59**. The U-4CR of **47c**, formaldehyde, methylamine, and phenylethyl isocyanide affords **59** in 52% yield (**Scheme 22**).
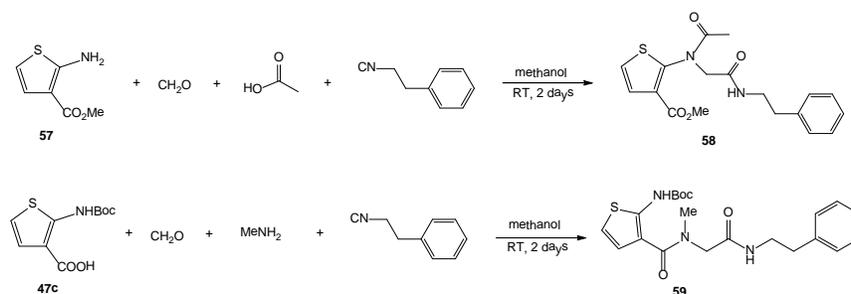
Scheme 22. Synthesis of dipeptide derivative via Ugi 4CR

α-Amino acids can react in an intramolecular version of the Ugi reaction (Ugi-5-center-4-component reaction, U-5C-4CR) in the presence of nucleophilic solvent, such like methanol.

However, β-amino acid derivatives were rarely been described to give the corresponding U-5C-4CR products.[235] Ugi described the reaction of anthranilic acids to yield different products depending on the substitution.[236] Herein, we investigated the U-5C-4CR of β-amino acid derivatives **60** derived from Gewald reaction. The corresponding U-5C-4CR products **61** were obtained using different substituted aldehydes and isocyanides (**Table 10**).

Table 10. Ugi-5-center-4-component reaction (U-5C-4CR)
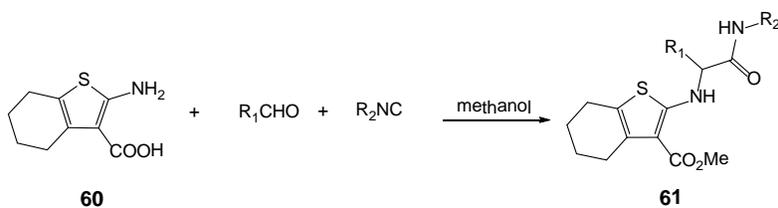
Entry	R^1	R^2	Yield (%)
a	^{i}Pr	^{t}Bu	51%
b	p-Cl-C$_6$H$_4$	^{t}Bu	18%
c	PhCH$_2$CH$_2$	^{t}Bu	47%
d	2-furanyl	^{t}Bu	19%
e	CH$_2$NHBoc	^{t}Bu	28%
f	^{i}Pr	cyclohexanyl	45%
g	^{i}Pr	benzyl	48%
h	^{i}Pr	CH$_2$CO$_2$Me	52%

Finally, we developed a synthetic method to allow rapid access to 1,4-thienodiazepines in just two steps from Gewald product. In the first step, the Gewald product **62** serves as an amine component for Ugi four-componment reaction with Boc glycine, isocyanide, and aldehyde. The

crude Ugi product **63** was treated with TFA in the presence of DCE to produce 1,4-thienodiazepine **64** (**Scheme 23**).
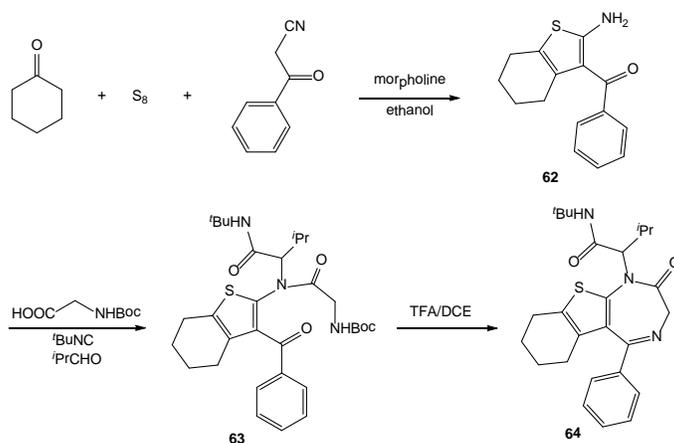
Scheme 23. Synthesis of 1,4-thienodiazepine

In summary, we have demonstrated that diverse aminothiophene scaffolds can be achieved by the IMCRs. The Gewald aminothiophenes were used as an amine component, an isocyanide component, as well as an acid component for MCRs. This approach enlarges the chemical space in maximal points of diversity offered by the G-3CR and the subsequent Ugi or Passerini reaction.

Materials and Methods

The starting materials derived from Gewald reaction were prepared according to the established methods.[125, 219] Preparative chromatography was conducted using preparative silica gel TLC plates (1000 μm, 20cm×20cm).

Methyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (46a): To a mixture of cyclohexanone (0.45 mol, 44.10 g), methyl cyanoacetate (0.3 mol, 28.77 g), sulfur (0.3 mol, 9.60 g), and 50 mL of ethanol, the solution of diethylamine (0.15 mol, 12.75 g) in 25 mL of ethanol

was added dropwise with magnetic stirring. After the completion of the addition, the reaction mixture was kept stirring under room temperate overnight. The reaction flask was kept in the freezer for crystallization. Then the precipitate was collected by vacuum filtration, and washed by cold ethanol. After air dry overnight, 42.20 g of yellow solid were obtained (yield: 67%). HPLC/MS: t_R = 10.58 min; m/z = 212.1 [M+H]$^+$ ^{1}H NMR (600 MHz, CDCl$_3$): 5.93 (2H, br.s, NH$_2$), 3.79 (3H, s, OMe), 2.67-2.69 (2H, m), 2.49-2.51 (2H, m), 1.73-1.78 (4H, m). ^{13}C NMR (150 MHz, CDCl$_3$): 166.5, 161.8, 132.4, 117.7, 105.6, 50.6, 26.9, 24.5, 23.3, 22.8.

2-Amino-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carbonitrile (46b): To a mixture of cyclohexanone (0.15 mol, 15.5 mL), malononitrile (0.1 mol, 6.61 g), sulfur (0.1 mol, 3.20 g) in 20 mL of ethanol under ice-water bath, diethylamine (0.05 mol, 6.04 mL) was added dropwise with magnetic stirring. After the completion of the addition, the reaction mixture was kept stirring under room temperate for 2 hours. The reaction flask was kept in the freezer for crystallization. Then the precipitate was collected by vacuum filtration, and washed by cold ethanol. After air dry overnight, 14.30 g of yellow solid were obtained (yield: 80%). HPLC/MS: t_R = 10.13 min; m/z = 178.9 [M+H]$^+$ ^{1}H NMR (600 MHz, CDCl$_3$): 4.63 (2H, br.s, NH$_2$), 2.50-2.52 (4H, m), 1.78-1.84 (4H, m). ^{13}C NMR (150 MHz, CDCl$_3$): 159.9, 132.4, 120.6, 115.5, 88.7, 24.5, 24.1, 23.4, 22.1.

Methyl 2-(*N*-(2-oxo-2-(phenethylamino)ethyl)acetamido)-4,5,6,7-tetrahydrobenzo[b] thiophene-3-carboxylate (49a): The mixture of **46a** (0.25 mmol, 52.8 mg), phenylethyl isocyanide (0.25 mmol, 45.6 mg), acetic acid (0.25 mmol, 14.3 μL), aqueous formic aldehyde (0.25 mmol, 18.6 μL), and 0.5 mL of methanol was stirring for 2 days under room temperature. The product was isolated by silica gel chromatography (hexanes/ethyl acetate, 1:3) as yellowish solid (43.7 mg, yield: 42%). HPLC/MS: t_R = 10.98 min; m/z = 414.9 [M+H]$^+$. HRMS:

$C_{22}H_{26}N_2O_4S$, 414.16133 (calcd.), 414.163269 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 7.28-7.30 (2H, m), 7.19-7.23 (3H, m), 6.94 (1H, br.s, NH), 4.32-4.35 (1H, m), 4.18-4.21 (1H, m), 3.77 (3H, s), 3.49-3.55 (2H, m), 2.83-2.84 (2H, m), 2.70-2.75 (4H, m), 1.98 (3H, s), 1.82-1.85 (4H, m). ^{13}C NMR (150 MHz, CDCl$_3$): 172.0, 168.2, 163.3, 148.2, 139.0, 135.1, 134.5, 128.8, 128.5, 126.4, 55.1, 51.9, 40.8, 35.5, 26.3, 25.1, 22.7, 22.4, 21.6.

N-(3-Cyano-4,5,6,7-tetrahydrobenzo[b]thiophen-2-yl)-N-(2-oxo-2-(phenethylamino) ethyl)acetamide (49b): The mixture of **46b** (0.25 mmol, 44.5 mg), phenylethyl isocyanide (0.25 mmol, 45.6 mg), acetic acid (0.25 mmol, 14.3 μL), aqueous formic aldehyde (0.25 mmol, 18.6 μL), and 0.5 mL of methanol was stirring for 2 days under room temperature. The product was isolated by silica gel chromatography (hexanes/ethyl acetate, 1:1) as yellowish solid (20.0 mg, yield: 21%). HPLC/MS: t_R = 10.81 min; m/z = 382.3 [M+H]$^+$. HRMS: $C_{21}H_{23}N_3O_2S$, 381.15110 (calcd.), 381.151931 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 7.29-7.32 (2H, m), 7.20-7.24 (3H, m), 6.10 (1H, br.s), 4.24 (2H, s), 3.53-3.56 (2H, m), 2.82-2.86 (2H, m), 2.68-2.70 (2H, m), 2.63-2.65 (2H, m), 2.08 (3H, s), 1.84-1.88 (4H, m). ^{13}C NMR (150 MHz, CDCl$_3$): 171.3, 167.6, 150.7, 138.6, 137.4, 134.4, 128.8, 128.6, 126.5, 113.0, 109.6, 54.0, 40.8, 35.4, 24.7, 24.3, 22.8, 21.8, 21.6.

Methyl 2-(1-(1-*tert*-butyl-1*H*-tetrazol-5-yl)-2-methylpropylamino)-4,5,6,7-tetrahydro benzo[b]thiophene-3-carboxylate (50a): The mixture of **46a** (0.3 mmol, 63.4 mg), isobutyladehyde (0.3 mmol, 27.4 μL), *tert*-butyl isocyanide (0.3 mmol, 33.9 μL), trimethylsilyl azide (0.6 mmol, 79.0 μL), and 0.5 mL of methanol was stirring for 2 days at 40 ^{o}C. The product was isolated by silica gel chromatography (hexanes/ethyl acetate, 5:1) as yellow solid (20 mg, yield: 17%). HPLC/MS: t_R = 12.78 min; m/z = 392.2 [M+H]$^+$. HRMS: $C_{19}H_{29}N_5O_2S$, 391.20420 (calcd.), 391.203323 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 8.25 (1H, d, J = 9.6 Hz), 4.72 (1H,

m), 3.79 (3H, s), 2.67-2.68 (2H, m), 2.49-2.52 (2H, m), 2.43-2.47 (1H, m), 1.80 (9H, s), 1.76-1.70 (2H, m), 1.71-1.75 (2H, m), 1.14 (3H, d, J = 6.6 Hz), 1.05 (3H, d, J = 6.6 Hz). ^{13}C NMR (150 MHz, CDCl$_3$): 166.5, 162.6, 154.6, 133.5, 116.9, 104.7, 61.8, 59.3, 50.8, 34.6, 30.5, 26.8, 24.5, 23.2, 22.7, 20.0, 17.8.

2-(1-(1-*tert*-Butyl-1H-tetrazol-5-yl)-2-methylpropylamino)-4,5,6,7-tetrahydrobenzo [b]thiophene-3-carbonitrile (50b): The mixture of **46b** (0.25 mmol, 44.5 mg), isobutyladehyde (0.25 mmol, 22.8 µL), *tert*-butyl isocyanide (0.25 mmol, 28.3 µL), trimethylsilyl azide (0.5 mmol, 65.8 µL), and 0.5 mL of methanol was stirring for 2 days at 40 $^{\circ}$C. The product was isolated by silica gel chromatography (hexanes/ethyl acetate, 5:1) as yellow solid (5.5 mg, yield: 6%). HPLC/MS: t_R = 11.90 min; m/z = 359.3 [M+H]$^+$. HRMS: C$_{18}$H$_{26}$N$_6$S, 358.19397 (calcd.), 358.193872 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 5.18 (1H, d, J = 10.2 Hz), 4.90 (1H, m), 2.48-2.53 (4H, m), 2.42 (1H, m), 1.82-1.83 (2H, m), 1.81 (9H, s), 1.78-1.79 (2H, m), 1.14 (3H, d, J = 6.6 Hz), 1.05 (3H, d, J = 6.6 Hz). ^{13}C NMR (150 MHz, CDCl$_3$): 160.2, 154.5, 133.5, 120.4, 115.5, 88.1, 61.9, 59.2, 34.8, 30.5, 24.4, 24.1, 23.2, 22.0, 19.9, 17.7.

2-Amino-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxamide (46c): 46b (10 mmol, 1.78 g) was added portion wise to 20 mL of concentrated sulfuric acid with stirring for 2 h. The reaction mixture was poured into ice-water, neutralized with potassium hydroxide. Then the solid were collected by vacuum filtration. The crude product was dissolved in DCM, washed by water. The organic layer was extracted by DCM, dried over anhydrous sodium sulfate. After the evaporation of the solvent, the product was obtained as yellow solid (1.40 g, yield: 71%). HPLC/MS: t_R = 7.81 min, m/z = 197.1 [M+H]$^+$ ^{1}H NMR (600 MHz, CD$_3$OD): 2.47-2.49 (2H, m), 2.42-2.47 (2H, m), 1.79-1.84 (4H, m). ^{13}C NMR (150 MHz, CD$_3$OD): 163.1, 131.3, 118.2, 115.7, 84.3, 24.1, 23.5, 23.2, 22.0.

N-*tert*-Butyl-2-(3-cyano-4,5,6,7-tetrahydrobenzo[b]thiophen-2-ylamino)-3-methyl butanamide (51): The mixture of **46c** (0.3 mmol, 58.8 mg), isobutyladehyde (0.3 mmol, 27.4 μL), *tert*-butyl isocyanide (0.3 mmol, 34.0 μL), acetic acid (0.3 mmol, 17.2 μL), and 0.5 mL of methanol was stirring overnight under room temperature. The mixture was added 10 mL of DCM, followed by 5% potassium hydroxide aqueous solution. The organic layer was extracted by DCM, dried over anhydrous sodium sulfate. After the evaporation of the solvent, the product was isolated by silica gel chromatography (hexanes/ethyl acetate, 5:1) as yellow solid (25 mg, yield: 25%). HPLC/MS: t_R = 11.82 min, m/z = 334.3 [M+H]$^+$. HRMS: $C_{18}H_{27}N_3OS$, 333.18748 (calcd.), 333.187204 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 5.98 (1H, br.s), 4.99 (1H, d, J = 6.0 Hz), 3.49 (1H, m), 2.50-2.54 (4H, m), 2.34 (1H, m), 1.78-1.85 (4H, m), 1.37 (9H, s), 1.04 (3H, d, J = 6.6 Hz), 1.01 (3H, d, J = 6.6 Hz). ^{13}C NMR (150 MHz, CDCl$_3$): 169.3, 162.0, 132.9, 120.4, 115.8, 87.1, 68.2, 51.6, 31.5, 28.7, 24.2, 23.3, 22.0, 19.3, 17.6.

Methyl 2-formamido-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (52): The mixture of **46a** (75 mmol, 15.85 g) and 50 mL of formic acid was stirring under reflux overnight. The reaction mixture was cooled to room temperature, then kept in the freezer for crystallization. The precipitate was collected by vacuum filtration, and washed by hexanes. The crude solid were dissolved in 200 mL of DCM, then neutralized by the saturated aqueous sodium bicarbonate (3 x 30 mL). After the extraction, the DCM layer was dried over anhydrous magnesium sulfate. After the evaporation, the product was obtained as yellow solid (16.30 g, yield: 91%). HPLC/MS: t_R = 10.89 min; m/z = 240.0 [M+H]$^+$. HRMS: $C_{11}H_{13}NO_3S$, 239.06161 (calcd.), 239.061233 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 11.20 (1H, br.s , NH), 8.48 (1H, s, CHO), 3.87 (3H, s, OMe), 2.74-2.76 (2H, m), 2.64-2.65 (2H, m), 1.78-1.79 (4H, m). ^{13}C NMR (150 MHz, CDCl$_3$): 166.8, 157.2, 145.4, 130.9, 127.5, 112.3, 51.5, 26.2, 24.3, 22.9, 22.7.

Methyl 2-isocyano-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (53): The mixture of **52** (10 mmol, 2.39 g) and THF (50 mL) was cooled to 0 $^{\circ}$C in nitrogen atmosphere. Triethylamine (55 mmol, 7.70 mL) was added and stirred for 5 min. Phosphorus chloride oxide (15 mmol, 1.37 mL) are added slowly to maintain the temperature at 0 $^{\circ}$C. The reaction mixture was allowed to keep stirring under room temperate overnight. Then the reaction mixture was poured into 100 mL of ice-water, and extracted by ether (3 x 100 mL). The organic layer was dried over anhydrous magnesium sulfate, and filtrated through a neutral aluminium oxide column. After the evaporation, the product was obtained as dark brown solid (2.15 g, yield: 97%). HPLC/MS: t_R = 11.86 min; m/z = 222.2 [M+H]$^+$. HRMS: $C_{11}H_{11}NO_2S$, 221.05105 (calcd.), 221.051681 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 3.92 (3H, s, OMe), 2.78-2.81 (2H, m), 2.69-2.71 (2H, m), 1.82-1.87 (4H, m). ^{13}C NMR (150 MHz, CDCl$_3$): 174.2, 161.8, 135.2, 134.9, 128.3, 51.9, 25.9, 24.9, 22.5, 22.1.

Methyl 2-(2-(benzylamino)-3-methylbutanamido)-4,5,6,7-tetrahydrobenzo[b] thiophene-3-carboxylate (54): The mixture of isobutyladehyde (0.3 mmol, 27.4 µL), benzylamine (0.3 mmol, 32.8 µL), **53** (0.3 mmol, 66.4 mg), phenyl phosphinic acid (0.03 mmol, 4.3 mg), and 0.5 mL of toluene was stirred for 3 days at 80 $^{\circ}$C. The product was isolated by silica gel chromatography (hexanes/ethyl acetate, 5:1) as yellow solid (25 mg, yield: 21%). HPLC/MS: t_R = 12.85 min; m/z = 401.0 [M+H]$^+$. HRMS: $C_{22}H_{28}N_2O_3S$, 400.18206 (calcd.), 400.180229 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 12.32 (1H, br.s, NH), 7.41-7.42 (2H, m), 7.33-7.36 (2H, m), 7.27-7.29 (1H, m), 3.92 (1H, ABd, J = 13.2 Hz), 3.89 (3H, s), 3.66 (1H, ABd, J = 13.2 Hz), 3.22 (1H, d, J = 4.8 Hz), 2.79-2.81 (2H, m), 2.67-2.69 (2H, m), 2.15-2.20 (1H, m), 1.79-1.83 (4H, m), 0.99 (3H, d, J = 7.2 Hz), 0.95 (3H, d, J = 7.2 Hz). ^{13}C NMR (150 MHz, CDCl$_3$): 171.7, 166.2, 146.8, 139.3,

131.0, 128.6, 128.5, 127.4, 126.6, 111.9, 67.88, 53.6, 51.33, 31.9, 26.3, 24.4, 23.0, 22.9, 19.4, 18.1.

Methyl 2-(2-acetoxy-3-methylbutanamido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (55): The mixture of isobutyladehyde (0.3 mmol, 27.4 μL), **53** (0.3 mmol, 66.4 mg), acetic acid (0.3 mmol, 17.2 μL), and 0.5 mL of DCM was stirring for 1 day under room temperature. The product was isolated by silica gel chromatography (hexanes/ethyl acetate, 3:1) as yellow solid (6 mg, yield: 6%). HPLC/MS: t_R = 12.78 min; m/z = 354.0 [M+H]$^+$. HRMS: $C_{17}H_{23}NO_5S$, 353.12969 (calcd.), 353.129559 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 11.82 (1H, br.s, NH), 5.32 (1H, d, J = 4.2 Hz), 3.88 (3H, s), 2.76-2.78 (2H, m), 2.66-2.67 (2H, m), 2.40-2.43 (1H, m), 2.33 (3H, s), 1.77-1.83 (4H, m), 1.03 (3H, d, J = 7.2 Hz), 0.99 (3H, d, J = 6.6 Hz). ^{13}C NMR (150 MHz, CDCl$_3$): 170.0, 166.94, 166.89, 146.5, 130.9, 127.1, 112.3, 51.4, 30.8, 29.7, 26.3, 24.4, 23.0, 22.8, 20.8, 18.7, 16.9.

1-(*tert*-Butylamino)-3-ethoxy-1,3-dioxopropan-2-yl 2-amino-4,5,6,7-tetrahydrobenzo [b]thiophene-3-carboxylate (56a): The mixture of **47a** (59.4 mg, 0.2 mmol), ethyl glyoxylate (0.2 mmol, 39.7 μL), *tert*-butyl isocyanide (0.2 mmol, 22.6 μL) in 0.5 mL of methanol was stirring under room temperate for 2 days. The reaction was quenched by water, and extracted by DCM. The organic layer was washed by saturated aqueous sodium bicarbonate, and dried over anhydrous sodium sulfate. After the evaporation of the solvent, the residue was treated by 1.0 mL of DCM (10% TFA), stirring under RT overnight. After the evaporation of the solvent, the product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 4:1) as yellow solid (20 mg, yield: 26%). HPLC/MS: t_R = 11.43 min; m/z = 383.1 [M+H]$^+$. HRMS: $C_{18}H_{26}N_2NaO_5S$, 405.14601 (calcd.), 405.1478 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 6.35 (1H, m), 6.16 (2H, m), 5.45 (1H, m), 4.31 (2H, m), 3.12 (1H, m), 2.85-2.88 (1H, m), 2.72 (1H, m),

2.51 (2H, m), 1.79 (5H, m), 1.39 (9H, s), 1.33 (3H, m). ^{13}C NMR (150 MHz, CDCl$_3$): 166.7, 164.2, 163.2, 162.7, 131.4, 118.0, 103.5, 72.8, 62.3, 51.9, 45.9, 28.6, 27.3, 24.4, 23.1, 22.6, 14.0, 8.6.

1-(*tert*-Butylamino)-3-ethoxy-1,3-dioxopropan-2-yl-2-amino-5-phenylthiophene-3-carboxylate (56b): The mixture of **47b** (63.8 mg, 0.2 mmol), ethyl glyoxylate (0.2 mmol, 39.7 µL), *tert*-butyl isocyanide (0.2 mmol, 22.6 µL) in 0.5 mL of methanol was stirring under room temperate for 2 days. The reaction was quenched by water, and extracted by DCM. The organic layer was washed by saturated aqueous sodium bicarbonate, and dried over anhydrous sodium sulfate. After the evaporation of the solvent, the residue was treated by 1.0 mL of DCM (10% TFA), stirring under RT overnight. After the evaporation of the solvent, the product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 4:1) as yellow solid (27 mg, yield: 33%). HPLC/MS: t_R = 11.34 min; m/z = 405.2 [M+H]$^+$. HRMS: C$_{20}$H$_{24}$N$_2$NaO$_5$S, 427.13036 (calcd.), 427.1269 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 7.25-7.42 (5H, m), 6.30 (3H, m), 5.54 (1H, m), 4.33 (2H, m), 3.87 (1H, m), 1.42 (9H, s), 1.34 (3H, m). ^{13}C NMR (150 MHz, CDCl$_3$): 166.7, 164.2, 162.7, 162.5, 133.7, 128.9, 126.8, 125.3, 124.8, 120.5, 105.6, 72.5, 62.5, 52.0, 28.6, 14.1.

Methyl 2-aminothiophene-3-carboxylate (57): Triethylamine (50 mmol, 5.0 mL) was added dropwise to a mixture of 1,4-dithiane-2,5-diol (7.60 g, 50 mmol), methyl cyanoacetate (9.59 g, 100 mmol), and DMF (40 mL). The mixture was stirred at 45 ^{o}C for 30 min. After cooled to RT, the reaction mixture was diluted with aqueous acetic acid (0.4 M, 200 mL). The mixture was extracted with ether (4 x 40 mL), and the combined organic layer was washed with water (2 x 40 mL). After dried over sodium sulfate, the organic layer was filtered through silica gel pad. After evaporation, 8.70 g of yellow solid were obtained (yield: 55%). HPLC/MS: t_R = 9.01 min; m/z =

158.2 [M+H]$^+$ ^{1}H NMR (600 MHz, CDCl$_3$): 6.98 (1H, d, J = 6.0 Hz), 6.20 (1H, d, J = 5.4 Hz), 5.94 (2H, br.s), 3.83 (3H, s). ^{13}C NMR (150 MHz, CDCl$_3$): 165.8, 162.7, 125.8, 107.0, 106.9, 51.0.

Methyl 2-(*N*-(2-oxo-2-(phenethylamino)ethyl)acetamido)thiophene-3-carboxylate (58): The mixture of **57** (0.2 mmol, 31.4 mg), phenylethyl isocyanide (0.2 mmol, 46 μL), acetic acid (0.2 mmol, 11.5 μL), aqueous formic aldehyde (0.2 mmol, 14.9 μL), and 0.5 mL of methanol was stirring for 2 days under room temperature. The product was isolated by silica gel chromatography (hexanes/ethyl acetate, 1:1) as yellow solid (13 mg, yield: 18%). HPLC/MS: t_R = 9.77 min; *m/z* = 383.1 [M+Na]$^+$. HRMS: C$_{18}$H$_{20}$N$_2$O$_4$S, 360.11438 (calcd.), 360.115030 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 7.29-7.35 (1H, m), 7.26-7.27 (2H, m), 7.20-7.22 (2H, m), 7.17-7.18 (2H, m), 4.45 (1H, ABd, J = 16.2 Hz), 4.24 (1H, ABd, J = 16.2 Hz), 3.80 (3H, s), 3.49-3.52 (2H, m), 2.79-2.87 (2H, m), 1.97 (3H, s). ^{13}C NMR (150 MHz, CDCl$_3$): 171.5, 167.8, 162.8, 161.1, 152.1, 139.0, 128.8, 128.5, 127.6, 126.4, 123.5, 55.2, 52.3, 40.9, 35.4, 21.4.

***tert*-Butyl 3-(methyl(2-oxo-2-(phenethylamino)ethyl)carbamoyl)thiophen-2-yl-carbamate (59):** The mixture of **47c** (0.2 mmol, 48.6 mg), phenylethyl isocyanide (0.2 mmol, 46 μL), methylamine (0.2 mmol, 17.6 μL), aqueous formic aldehyde (0.2 mmol, 14.9 μL), and 0.5 mL of methanol was stirring for 2 days under room temperature. The product was isolated by silica gel chromatography (hexanes/ethyl acetate, 1:1) as yellow solid (43 mg, yield: 52%). HPLC/MS: t_R = 10.93 min; *m/z* = 418.0 [M+H]$^+$. HRMS: C$_{21}$H$_{27}$N$_3$O$_4$S, 417.17223 (calcd.), 417.173325 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 9.83 (1H, s), 7.24-7.28 (2H, m), 7.16-7.20 (3H, m), 6.83 (1H, s), 6.70 (1H, m), 6.46 (1H, s), 4.02 (2H, s), 3.59 (2H, m), 3.08 (3H, s), 2.85 (2H, m), 1.53 (9H, s).^{13}C NMR (150 MHz, CDCl$_3$): 168.6, 168.4, 152.3, 149.3, 138.5, 128.7, 128.6, 126.6, 123.5, 114.7, 112.9, 81.9, 40.4, 35.5, 28.3.

Preparation of **2-amino-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid (60)**: To a mixture of **46a** (10 mmol, 2.11 g) in 20 mL of ethanol, KOH (40 mmol, 2.24 g) in 20 mL of water was added and the reaction was reflux for 5 h. After cooling, the reaction mixture was diluted with water and washed by ether. The aqueous solution was adjusted to pH = 6 with 1 M HCl aq. The product was collected by vacum filtration (yellow solid, 1.50 g, 76%). HPLC/MS: 1.86 min, $[M+H]^+$: 198.2 ^{1}H NMR (600 MHz, CD$_3$OD): 2.68-2.70 (2H, m), 2.47-2.49 (2H, m), 1.74-1.80 (4H, m). ^{13}C NMR (150 MHz, CD$_3$OD): 167.8, 163.7, 132.2, 116.2, 103.8, 26.5, 24.0, 23.1, 22.6.

General procedure for the synthesis of compounds 61:

Preparation of **methyl 2-(1-(*tert*-butylamino)-3-methyl-1-oxobutan-2-ylamino)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (61a)**: The mixture of **60** (0.2 mmol, 49.3 mg), isobutyladehyde (0.2 mmol, 22.8 μL), *tert*-butyl isocyanide (0.2 mmol, 28.3 μL), and 0.5 mL of methanol was stirring for 2 days under room temperature. The product was isolated by silica gel chromatography (hexanes/ethyl acetate, 3:1) as yellowish solid (37 mg, yield: 51%). HRMS: C$_{19}$H$_{30}$N$_2$O$_3$S, 366.197715 (calcd.), 366.196604 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 8.03 (1H, d, J = 6.0 Hz), 6.29 (1H, s), 3.82 (3H, s), 3.49 (1H, m), 2.71-2.72 (2H, m), 2.52-2.54 (2H, m), 2.47-2.50 (1H, m), 1.74-1.80 (4H, m), 1.37 (9H, s), 1.03 (3H, d, J = 7.2 Hz), 1.00 (3H, d, J = 6.6 Hz). ^{13}C NMR (150 MHz, CDCl$_3$): 170.2, 167.2, 164.8, 132.8, 118.3, 104.7, 68.9, 51.1, 50.8, 30.9, 28.6, 26.8, 24.6, 23.2, 22.7, 19.8, 16.8.

Preparation of **methyl 2-(2-(*tert*-butylamino)-1-(4-chlorophenyl)-2-oxoethylamino)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (61b)**: The product was isolated by silica gel chromatography (hexanes/ethyl acetate, 3:1) as yellow solid (16 mg, yield: 18%). HRMS: C$_{22}$H$_{28}$N$_2$O$_3$SCl, 435.1509 (calcd.), 435.1521 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.33 (s, 9H),

1.73-1.76 (m, 4H), 2.51 (m, 2H), 2.71 (m, 2H), 3.81 (s, 3H), 4.64 (d, 1H, J = 4.8 Hz), 5.93 (s, 1H), 7.36-7.17 (m, 4H), 8.51 (d, 1H, J = 4.8 Hz). ^{13}C NMR (150 MHz, CDCl$_3$): 22.7, 23.2, 24.6, 26.7, 28.5, 50.8, 51.7, 65.6, 105.3, 118.5, 128.7, 129.4, 133.0, 134.5, 136.5, 161.9, 166.7, 168.0.

Preparation of **methyl 2-(1-(*tert*-butylamino)-1-oxo-4-phenylbutan-2-ylamino)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (61c)**: The product was isolated by silica gel chromatography (hexanes/ethyl acetate, 3:1) as yellow solid (40 mg, yield: 47%). HPLC/MS: t_R = 13.26 min; m/z = 429.4 [M+H]$^+$. HRMS: C$_{24}$H$_{32}$N$_2$O$_3$SNa, 451.2031 (calcd.), 451.2067 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.35 (s, 9H), 1.78-1.81 (m, 4H), 2.07 (m, 1H), 2.36 (m, 1H), 2.56(m, 2H), 2.72-2.75 (m, 3H), 2.83 (m, 1H), 3.59 (m, 1H), 3.85 (s, 3H), 6.27 (s, 1H), 7.21-7.22 (m, 3H), 7.28-7.29 (m, 2H), 8.00 (d, 1H, J = 5.4 Hz). ^{13}C NMR (150 MHz, CDCl$_3$): 22.8, 23.2, 24.6, 26.8, 28.6, 32.2, 35.2, 50.8, 51.2, 62.9, 105.0, 118.6, 126.2, 128.50, 128.53, 132.8, 140.6, 163.7, 167.2, 170.7.

Preparation of **methyl 2-(2-(*tert*-butylamino)-1-(furan-2-yl)-2-oxoethylamino)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (61d)**: The product was isolated by silica gel chromatography (hexanes/ethyl acetate, 3:1) as yellow solid (15 mg, yield: 19%). HRMS: C$_{20}$H$_{26}$N$_2$O$_4$SNa, 413.1511 (calcd.), 413.1514 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.35 (s, 9H), 1.72-1.80 (m, 4H), 2.45-2.75 (m, 4H), 3.62 (s, 3H), 4.30 (s, 1H), 5.95 (m, 1H), 6.14 (m, 1H), 6.24 (s, 1H), 6.39 (m, 1H), 6.95 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 22.2, 23.1, 24.4, 25.6, 28.3, 52.0, 57.6, 61.1, 92.0, 112.9, 114.2, 121.1, 129.2, 130.1, 132.4, 163.6, 165.4, 184.4.

Preparation of **methyl 2-(3-(*tert*-butoxycarbonylamino)-1-(*tert*-butylamino)-1-oxopropan-2-ylamino)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (61e):** The product was isolated by silica gel chromatography (hexanes/ethyl acetate, 3:1) as yellow solid (25 mg, yield: 28%). HPLC/MS: t_R = 12.40 min; m/z = 454.3 [M+H]$^+$. HRMS: C$_{22}$H$_{35}$N$_3$O$_5$SNa, 476.2195 (calcd.),

476.2218 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.35 (s, 9H), 1.46 (s, 9H), 1.75-1.79 (m, 4H), 2.54-2.73 (m, 4H), 3.60-3.75 (m, 3H), 3.82 (s, 3H), 5.06 (s, 1H), 6.42 (s, 1H), 8.08 (m, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 22.7, 23.2, 24.6, 26.8, 28.4, 28.6, 43.0, 50.8, 51.5, 63.9, 80.0, 105.5, 118.3, 133.5, 156.5, 162.7, 166.2, 169.0.

Preparation of **methyl 2-(1-(cyclohexylamino)-3-methyl-1-oxobutan-2-ylamino)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (61f)**: The product was isolated by silica gel chromatography (hexanes/ethyl acetate, 3:1) as yellow solid (35 mg, yield: 45%). HPLC/MS: t_R = 13.01 min; m/z = 393.2 [M+H]$^+$. HRMS: C$_{21}$H$_{32}$N$_2$O$_3$SNa, 415.2031 (calcd.), 415.2054 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.00 (3H, d, J = 6.6 Hz), 1.05 (3H, d, J = 7.2 Hz), 1.08-1.37 (m, 7H), 1.62-1.89 (m, 9H), 2.52-2.54 (m, 3H), 2.72 (m, 2H), 3.59 (m, 1H), 3.80 (m, 1H), 3.83 (s, 3H), 6.38 (1H, d, J = 8.4 Hz), 8.05 (1H, d, J = 6.6 Hz). ^{13}C NMR (150 MHz, CDCl$_3$): 16.7, 19.9, 22.7, 23.2, 24.6, 24.86, 24.91, 25.5, 26.8, 31.0, 32.9, 33.2, 48.2, 50.9, 68.4, 104.7, 118.4, 132.8, 164.8, 167.3, 170.0.

Preparation of **methyl 2-(1-(benzylamino)-3-methyl-1-oxobutan-2-ylamino)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (61g)**: The product was isolated by silica gel chromatography (hexanes/ethyl acetate, 3:1) as yellow solid (38 mg, yield: 48%). HPLC/MS: t_R = 12.46 min; m/z = 401.3 [M+H]$^+$. HRMS: C$_{22}$H$_{28}$N$_2$O$_3$SNa, 423.1718 (calcd.), 423.1721 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.02 (3H, d, J = 6.6 Hz), 1.07 (3H, d, J = 6.6 Hz), 1.75-1.80 (m, 4H), 2.53-2.59 (m, 3H), 2.71 (m, 2H), 3.73 (m, 1H), 3.78 (s, 3H), 4.39-4.42 (m, 1H), 4.54-4.58 (m, 1H), 6.90 (m, 1H), 7.23-7.33 (m, 5H), 8.10 (1H, d, J = 6.6 Hz). ^{13}C NMR (150 MHz, CDCl$_3$): 16.9, 19.9, 22.8, 23.2, 24.6, 26.8, 31.0, 43.4, 50.9, 68.3, 104.7, 118.3, 127.5, 127.7, 128.6, 133.0, 138.0, 164.5, 167.3, 171.3.

Preparation of **methyl 2-(1-(2-methoxy-2-oxoethylamino)-3-methyl-1-oxobutan-2-ylamino)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (61h)**: The product was isolated by silica gel chromatography (hexanes/ethyl acetate, 3:1) as yellow solid (40 mg, yield: 52%). HPLC/MS: t_R = 11.63 min; m/z = 383.0 [M+H]$^+$. HRMS: $C_{18}H_{27}N_2O_5S$, 383.1641 (calcd.), 383.1810 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.5-1.087 (6H, m), 1.74-1.78 (m, 4H), 2.53 (m, 2H), 2.71 (m, 2H), 3.69 (m, 1H), 3.74 (s, 3H), 3.81 (s, 3H), 3.90-3.94 (m, 1H), 4.16-4.20 (m, 1H), 7.00 (m, 1H), 8.13 (1H, d, J = 6.0 Hz). ^{13}C NMR (150 MHz, CDCl$_3$): 16.8, 19.8, 22.7, 23.2, 24.6, 26.8, 31.1, 41.0, 50.9, 52.4, 68.0, 104.7, 118.2, 133.0, 164.4, 167.2, 170.0, 171.9.

Preparation of **(2-amino-4,5,6,7-tetrahydrobenzo[b]thiophen-3-yl)(phenyl)methanone (62)**: To a mixture of cyclohexanone (10 mmol, 1.03 mL), cyanoacetate (10 mmol, 1.45 g), sulfur (10 mmol, 0.32 g), and 10 mL of ethanol, morpholine (10 mmol, 0.87 mL) was added dropwise with magnetic stirring. After the completion of the addition, the reaction mixture was kept stirring under 70 ^{o}C overnight. The mixture was evaporated and the residue diluted with dichloromethane. After the mixture was washed with water, the organic layer was dried over Na$_2$SO$_4$ and filtered. After evaporation of the solvent, 2.3 g of yellow solid were obtained (yield: 89%). HPLC/MS: t_R = 11.52 min; m/z = 258.0 [M+H]$^+$ ^{1}H NMR (600 MHz, CDCl$_3$): 1.49-1.51 (m, 2H), 1.74-1.84 (m, 4H), 2.53-2.56 (m, 2H), 6.69 (br.s, 2H), 7.41-7.51 (m, 5H).

Preparation of ***tert*-butyl 2-((3-benzoyl-4,5,6,7-tetrahydrobenzo[b]thiophen-2-yl)(1-(*tert*-butylamino)-3-methyl-1-oxobutan-2-yl)amino)-2-oxoethylcarbamate (63)**: The mixture of **62** (0.2 mmol, 51.4 mg), 2-(tert-butoxycarbonylamino)acetic acid (0.2 mmol, 35.0 mg), tert-butyl isocyanide (0.2 mmol, 28.3 μL), isobutyraldehyde (0.2 mmol, 22.8 μL) and 0.5 mL of methanol was stirring for 2 days under room temperature. The product was isolated by silica gel chromatography (hexanes/ethyl acetate, 5:1) as yellowish solid (26 mg, 23%). HPLC/MS: t_R =

13.26 min; m/z = 570.2 [M+H]$^+$. HRMS: $C_{31}H_{43}N_3O_5SNa$, 592.2821 (calcd.), 592.2814 (found).

^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 0.88-0.93 (m, 8H), 0.93 (s, 9H), 1.06 (s, 6H), 1.46 (s, 9H), 1.47 (s, 6H), 1.70-1.72 (m, 4H), 1.88-2.11 (m, 6H), 2.38-2.41 (m, 1H), 2.55-2.59 (m, 1H), 2.74-2.76 (m, 4H), 3.60 (m, 1H), 3.92-3.96 (m, 1H), 4.02-4.06 (m, 1H), 4.30-4.43 (m, 3H), 5.40 (s, 1H), 5.45 (s, 1H), 5.89 (s, 1H), 7.06 (s, 1H), 7.40-7.46 (m, 4H), 7.51-7.57 (m, 2H), 7.72-7.73 (m, 2H), 7.76-7.77 (m, 2H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 19.4, 19.9, 20.5, 22.2, 22.4, 22.8, 22.9, 24.9, 26.0, 26.9, 27.4, 27.8, 28.1, 28.4, 43.8, 44.1, 50.7, 51.0, 79.4, 79.6, 128.0, 128.4, 129.7, 130.2, 132.4, 132.8, 133.4, 136.1, 136.7, 137.6, 137.7, 138.0, 138.1, 138.3, 155.8, 167.6, 168.7, 172.8, 191.9, 192.7.

Preparation of **N-*tert*-butyl-3-methyl-2-(2-oxo-5-phenyl-2,3-dihydro-1*H*-(6,7,8,9-tetrahydro)-benzothieno[2,3-e][1,4]diazepin-1-yl)butanamide (64)**: The mixture of **63** (26 mg), and 0.5 mL of DCE (10% TFA) was stirring under 40 ^{o}C overnight. The residue was treated by triethylamine (100 µL), and the product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 3:1) as yellowish solid (18 mg, yield: 87%). HPLC/MS: t_R = 12.67 min; m/z = 452.0 [M+H]$^+$. HRMS: $C_{26}H_{34}N_3O_2S$, 452.2372 (calcd.), 452.2398 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 0.63 (d, 3H, J = 6.6 Hz), 0.86 (d, 1H, J = 6.6 Hz), 1.00 (d, 3H, J = 6.6 Hz), 1.02 (d, 1H, J = 6.6 Hz), 1.24 (s, 2H), 1.37 (s, 9H), 1.46 (m, 1H), 1.70-1.87 (m, 7H), 2.65-2.74 (m, 4H), 3.98 (d, 1H, J = 10.2 Hz), 4.42 (d, 1H, J = 11.4 Hz), 4.88 (d, 1H, J = 10.8 Hz), 6.28 (s, 1H), 7.40-7.48 (m, 4H), 7.57-7.61 (m, 3H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 19.1, 20.0, 22.5, 23.0, 24.5, 26.1, 28.5, 28.6, 51.5, 58.0, 128.5, 128.7, 130.4, 130.5, 132.3, 137.8, 167.8, 168.8.

3.3 DESIGN AND SYNTHESIS OF 1,4-THIENODIAZEPINE LIBRARIES

Based on the above discussion, IMCR was applied to generate pilot-scale libraries for drug discovery purpose, preferentially targeting PPIs. In **Chapter 3.3.1**, a small focused compound library of TDZs was screened, and some compounds exhibited promising activity as p53-Mdm2 inhibitors. In **Chapter 3.3.2**, a second scaffold of TDZs was designed to synthesize drug-like compounds incorporating "anchor" residues, which are aboundant in the PPI interface.

3.3.1 Synthesis, Virtual Space and p53-Mdm2 Activity*

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Thiophene as an effective bioisostere of phenyl ring led to the discovery of a series of 1,4-thienodiazepine drugs, such as the block buster family of olanzapine, clotiazepam, and brotizolam. For example, clotiazepam is structurally similar to diazepam, which is one of the most frequently prescribed medications in the world during the past 40 years.[237] Thus, the development of new thiophene scaffolds based on privileged structures, especially 1,4-benzodiazepine family is of great interest.

MCR is an alternative strategy in different drug discovery stages including lead discovery and pre-clinical process development.[238] MCRs allow the resource and cost effective, fast, and convergent synthesis of diverse compound libraries, and highly improve the efficiency to explore the chemical space with limited synthetic effort.[239] The Gewald-3CR (G-3CR) is a unique method using elemental sulfur to yield thiophene ring, which builds a platform for the synthesis of new thiophene scaffolds.[209] For example, olanzapine is manufactured by Eli Lilly via the

formation of its thiophene ring through G-3CR.[240] G-3CR provides a convenient way to synthesize 2-aminothiophene **66**, which is bioisostere to anthranilic acid **65** (**Figure 34**).[214] According to the substructure search in currently published protein-ligand cocrystal structures (PDB query and Relibase[241]), most of the retrieved ligands are nonsubstituted anthranilic acid derivatives. Advantageously, the G-3CR accessible thiophene scaffold allows for many more synthetic variations than the substituted anthranilic acids derivatives.
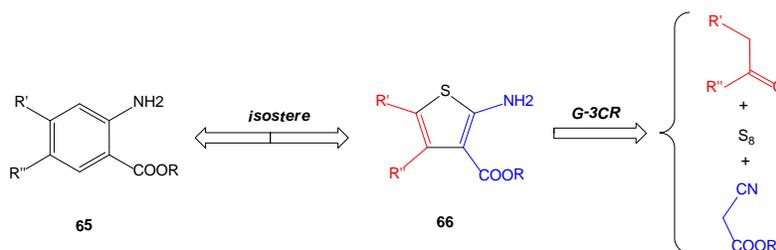
Figure 34. Gewald aminothiophene as a bioisostere of anthranilic acids

IMCRs, such as the Ugi and Passerini reactions, provide a powerful tool for producing arrays of compounds based on multiple scaffolds and with high atom economy.[242] The UDC approach generates very useful classes of scaffolds amenable by an initial Ugi reaction of two bifunctional orthogonally protected starting material classes and a secondary reaction to form heterocyclic rings, such as benzimidazole, quinoxalinone, imidazoline, γ-lactame, ketopiperazine and diketopiperazine.[243] Notably, UDC strategy is particularly useful for the synthesis of 1,4-benzodiazepine-2,5-diones.[244-247] The further enlargement of UDC amenable scaffold classes is highly desirable, and therefore we investigated the development of synthetic routes to TDZs. Herein, we report the preparation of a new TDZ scaffold by a union of Gewald and UDC MCRs (**Figure 35**).
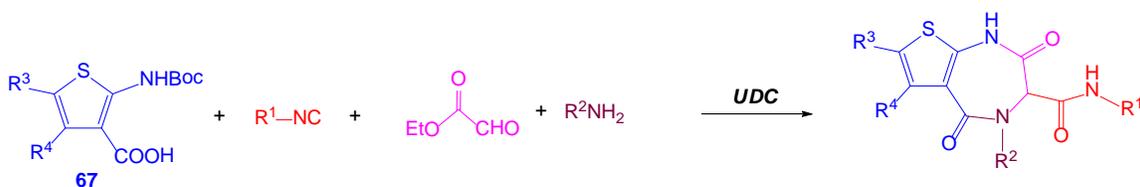
Figure 35. Synthesis of the TDZ scaffold via the UDC approach (R^1, R^2, R^3, R^4: points of diversity)

Synthesis of 1,4-thienodiazepine-2,5-dione scaffold

The synthesis of the thienodiazepinedione scaffold **69** was designed by employing the Ugi-4CR and a subsequent deprotection and ring closure. In order to access a new scaffold with high variability the thiophene carboxylic acid was synthesized by another versatile MCR the Gewald-3CR. We synthesized three different exemplary substituted thiophene carboxylic acids to investigate scope and limitations and the influence of this particular starting material class in the subsequent UDC procedure. Cyclohexane-fused aminothiophene **66a** was prepared via the G-3CR of cyclohexanone, sulfur and methyl cyanoacetate.[221] 4,5-Unsubstituted aminothiophene **66b** was synthesized from the Gewald reaction of 1,4-dithiane-2,5-diol and methyl cyanoacetate.[234] Phenyl-substitued aminothiophene **66c** was obtained from the G-3CR of phenylacetaldehyde, sulfur and methyl cyanoacetate.[234] Then we initiated the preparation of thiophene carboxylic acid **67a-c** by Boc-protection of **66a-c**, followed by hydrolysis (**Scheme 24**). After pH adjustment to 6, compounds **67a-c** were precipitated out and collected by filtration, which are ready to be applied according to UDC strategy.

66a, R^3, R^4 = (-CH$_2$-)$_4$
66b, R^3, R^4 = H
66c, R^3 = Ph, R^4 = H

67a, R^3, R^4 = (-CH$_2$-)$_4$; 48%
67b, R^3, R^4 = H; 31%
67c, R^3 = Ph, R^4 = H; 47%
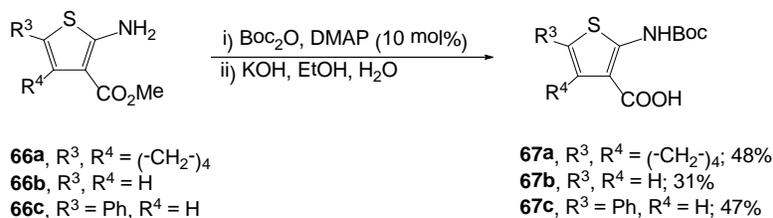
Scheme 24. Synthesis of **67a-c**.

Therefore, *N*-Boc thiophene carboxylic acid **67a** was employed as the bifunctional mono protected starting material for the synthesis of TDZs **69a**. Interestingly, there is no previous report on the compatibility of Gewald aminothiophene derivatives employed in the Ugi-4CR. A preliminary investigation by the reaction of thiophene carboxylic acid **67a**, benzylamine, *tert*-butyl isocyanide, and ethyl glyoxalate, gave the desired condensation product **68a-1** in 65% yield (**Scheme 25**). The previous work in our lab demonstrated that TBD is an efficient organocatalyst for amidations of primary and secondary amines under mild conditions.[248] As expected, compound **69a-1** was obtained by the deprotection of **68a-1** under a 10% solution of TFA in dichloromethane and following cyclization using catalytic amount of TBD in 62% yield over two steps.
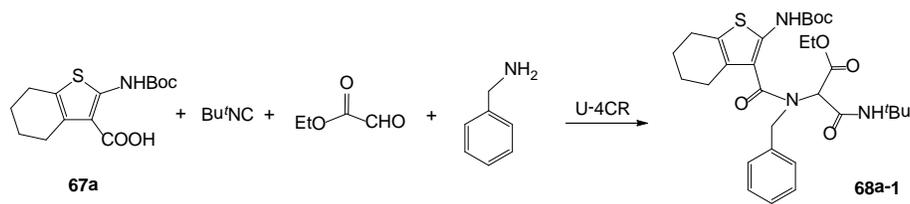
Scheme 25. Ugi-4CR of **67a.** Regents and conditions: MeOH, RT, 2 days, 65%.

Under further investigation, we found that it is not necessary to isolate the Ugi product as the intermediate for next steps. The following deprotection of the crude Ugi product and subsequent cyclization gave the corresponding thienodiazepinedione **69a-1** in 33% yield over three steps. Encouraged by these results, a small focused library of thienodiazepinediones **69a** was synthesized in order to enlarge the diversity of reactants (**Table 11**). The thienodiazepinedione products were isolated by chromatography without isolation of the intermediate. This protocol works well by employing different substituted amines and isocyanides as well. Overall, the yield is around 50-80% in average for each step of the

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transformation. Thus we predict that the procedure isolating and purifying (e.g. by mass directed HPLC) only the final product will be useful for automated library generation for the production of large libraries.

Table 11. Synthesis of 1,4-thienodiazepine-2,5-diones **69a**
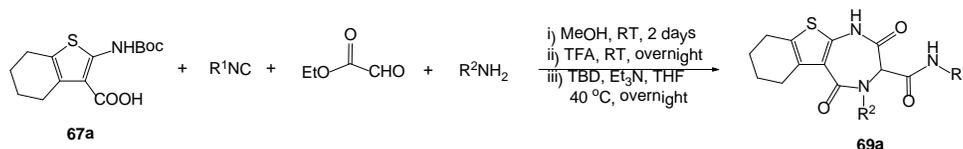
entry	R^1	R^2	compound	yield (%)^a
1	t-Bu	benzyl	**69a-1**	33
2	t-Bu	2-phenyl ethyl	**69a-2**	53
3	t-Bu	4-chlorobenzyl	**69a-3**	28
4	t-Bu	4-chlorophenyl	**69a-4**	16
5	cyclopropyl methyl	benzyl	**69a-5**	26
6	$2,4,6$-$(CH_3)_3C_6H_2$	4-chlorobenzyl	**69a-6**	12
7	benzyl	4-chlorobenzyl	**69a-7**	16
8	3,4-dichlorobenzyl	4-chlorobenzyl	**69a-8**	16

^{a} isolated yields, over three steps (Method A).

The Ugi reaction of *N*-Boc thiophene carboxylic acid **67b**, 2-phenyl ethylamine, *tert*-butyl isocyanide, and ethyl glyoxalate, gave the corresponding condensation product **68b-1** in 56% yield, isolated by chromatography. A mild condition was adopted for the deprotection of Ugi product **69b-1** using a 10% solution of TFA in dichloromethane. However, the 'three-step, one-separation' procedure for the synthesis of **69b-1** is unsatisfactory by using the crude Ugi product without separation. The transformation of **68b-1** after purification afforded the

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corresponding TDZ **69b-1**. *N*-Boc thiophene carboxylic acid **67b** was employed as the bifunctional starting material for the synthesis of TDZs **69b** under UDC sequence (**Table 12**). Meanwhile, TDZs **69c** were synthesized from *N*-Boc thiophene carboxylic acid **3c** under similar conditions. The Ugi reaction of **3c** and ethyl glyoxalate with different amines and isocyanides gave the condensation products **68c** in 38-70% yield. Then, a series of TDZs **69c** were obtained by deprotection and subsequent cyclization of the corresponding Ugi products (**Table 12**).

Table 12. UDC approach for the synthesis of **69b** and **69c**
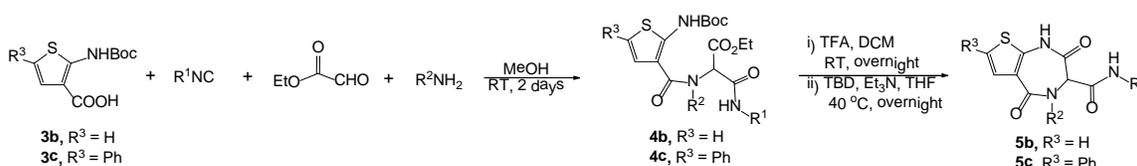
entry	R^1	R^2	compound	yield (%)^a
1	*t*-Bu	2-phenyl ethyl	**68b-1**	56
			69b-1	22^b
2	*t*-Bu	3-thienyl ethyl	**68b-2**	45
			69b-2	43^b
3	cyclohexyl	benzyl	**68b-3**	52
			69b-3	20^b
4	cyclopropyl methyl	3,4-dimethoxy phenyl ethyl	**68b-4**	36
			69b-4	38^b
5	*t*-Bu	cyclopropyl methyl	**68c-1**	41
			69c-1	35^c
6	*t*-Bu	2-methoxy ethyl	**68c-2**	49
			69c-2	27^c
7	*t*-Bu	phenyl ethyl	**68c-3**	52
			69c-3	38^c
8	cyclopropyl methyl	2-methoxy ethyl	**68c-4**	38
			69c-4	29^c
9	cyclohexyl	2-methoxy ethyl	**68c-5**	70
			69c-5	19^c
10	cyclohexyl	cyclopropyl methyl	**68c-6**	60
			69c-6	25^c

^{a} Isolated yields; ^{b} over two steps (Method B); ^{c} over two steps (Method C).

In summary, we have demonstrated that TDZ scaffold can be achieved by the union of two sequential MCRs.[249] This approach takes advantages of the maximal points of diversity offered by the G-3CR and U-4CR. A wide range of amines and isocyanides, as well as thiophene carboxylic acids are tolerated under the reaction procedure. All compounds are racemic, because the Ugi reaction yields a new stereocenter at C-3 position. Our synthetic strategy allows convenient preparation of these compounds to avoid the limitations imposed by traditional methods involving the condensation of amino acid derivatives.[250]

Cheminformatics of virtual libraries

In order to evaluate the chemical space of TDZ scaffold, a virtual library was created based on the synthetic route. We developed a random module software for the program Reactor (JChem 5.2.2, 2009, www.chemaxon.com) allowing us to generate a random virtual library (N = 50,000). The compounds from the virtual library were introduced into Instant JChem (Instant JChem 2.5.1, 2009, www.chemaxon.com) to calculate some main physical properties, such as rule-of-five parameters. In addition, the compound library of commercially available benzodiazepines (substructure search from eMolecules, N = 2,498) was also analyzed by Instant JChem for comparison. The distribution of benzodiazepine library and a random virtual library of TDZs (N = 50,000) was presented in term of the major descriptors molecular weight, logP and total polar surface area (TPSA) in **Figure 36**. The virtual library based on this new scaffold enlarges the chemical space of the benzodiazepine family. We foresee this scaffold to be useful in virtual screening and lead discovery, due to its largely unexplored chemical space.
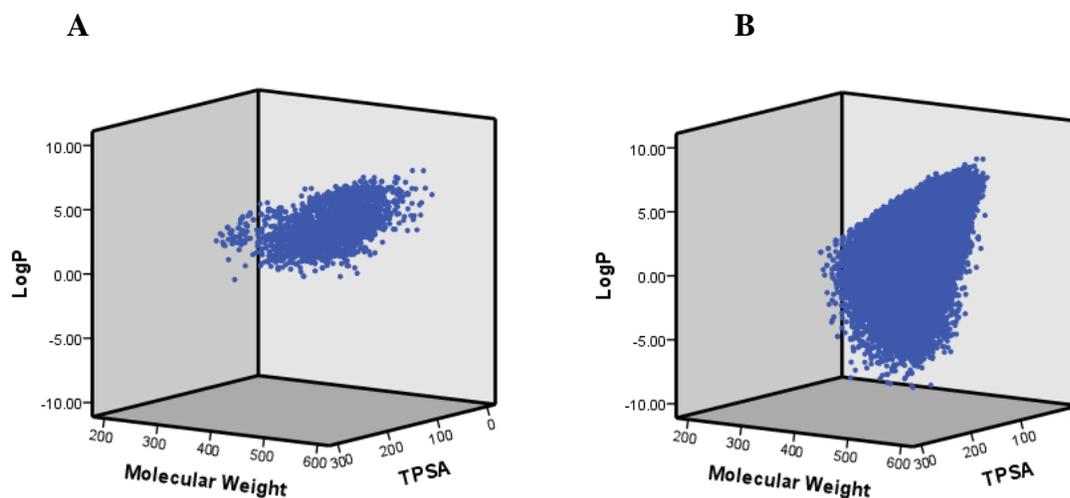
Figure 36. The distribution of molecular weight, logP and TPSA: (A) benzodiazepine library of a substructure search from eMolecules (N = 2,498); (B) a random virtual library of 1,4-thienodiazepine-2,5-diones (N = 50,000).

The physical properties of the virtual library were analyzed by frequency distributions in PASW Statistics 18. The range of molecular weight (between 253.3 Da. and 569.7 Da. with a mean value of 469.7 Da.) is broad due to the diversity of reactant components. The molecular weight of the compounds we physically synthesized is less than 500 Da. The mean of logP is 2.1 with standard deviation 2.3 indicating drug-like properties. TPSA is a major descriptor relevant for example for cell membrane permeability.[251] TPSA of the majority of compounds is between 126.8 Å^2 and 190.4 Å^2. The number of rotatable bonds (NRB), a very good descriptor of oral bioavailability, has a mean value of 7 with the standard deviation 2. The number of HBA (hydrogen bond acceptors) ranges from 4 to 13, with an average number of 7. The number of HBD (hydrogen bond donors) ranges from 2 to 8, with an average number of 3. In terms of drug likeness, 67.0% of 50,000 compounds obey Lipinski's rule. And 85.8% of them are predicted to be bioavailable (mass $\leq$ 500, LogP $\leq$ 5, HBD $\leq$ 5, HBA $\leq$ 10, PSA $\leq$ 200, NRB $\leq$ 10, and fused aromatic rings $\leq$ 5). Noteworthy, the overall shape of the compounds based on the thienodiazepinedione scaffold is non-planar. The presence of stereochemistry and sp^3 centers has

been recognized to correlate with success as compounds transition from discovery, through clinical testing, to drugs.[252] Based on the commonly accepted descriptors, it is likely that lead-like compounds with novel and potent biological activity will be obtained based on this scaffold.

We also create a virtual library of 3-D conformers, which can be used as an input for docking and other virtual analysis software. Omega (Omega 2.3.2, 2009, www.eyesopen.com) was used to generate 3-D structures of a random virtual library (N = 5,000). For example, the tricyclic backbone (6-, 5-, 7-membered rings) in 3-D structures of compound **69a-7** has the same conformation, while two side chains are oriented oppositely with certain rotation flexibility.

Inhibitor activities of p53-Mdm2 interaction

Since quite sometimes we are interested in the design of small molecular weight p53-Mdm2 inhibitors and new scaffolds desirable for potent and selective candidates.[123, 153, 253] The tumor suppressor p53 is well recognized as a therapeutic target for new anticancer interventions.[254] The activation of wild-type p53 in human tumors by antagonizing murine double minute 2 (Mdm2) is a promising and potentially non toxic therapeutic strategy.[82] The disruption of the p53-Mdm2 interaction can be accomplished by mimicking the p53 fragment with particular emphasis on the Mdm2 binding site using peptides, foldamers and peptoids (α-helical transactivation domain).[255] However, small-molecule libraries are favorable for the design of Mdm2 inhibitors in terms of the desirable bioavailability and stability.[153] The first class of potent and selective small-molecule Mdm2 inhibitors, nutlins were identified from *cis*-imidazoline compounds.[28] Next, benzodiazepinedione compound **70** has been identified as a potent Mdm2 inhibitor (K_d = 80 nM).[27] The cocrystal structure of Mdm2 complex shows that compound **70** binds to the p53-binding site (side chains Phe 19, Trp 23, and Leu 26) of Mdm2

(**Figure 37**). Such benzodiazepinedione inhibitors were found to suppress human tumor cell proliferation *in vitro* and sensitize tumors to doxorubicin *in vivo*.[256] Further optimization and SAR were investigated in order to improve the potency and cellular activity of BDZ inhibitors.[86, 257-259] Although bioisosterism is a lead modification approach to attenuate the biological properties of the lead compounds, TDZ scaffold as a potential pharmacophore has been rarely investigated so far. Thus we hypothesized that the peptidomic TDZ scaffold could also act as α-helix mimetics to disturb the p53-Mdm2 interaction.
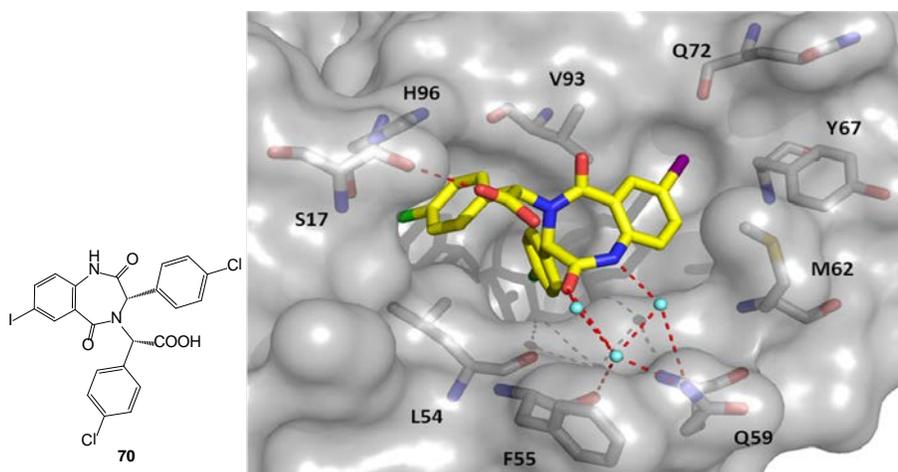
Figure 37. Cocrystal structure of benzodiazepinedione Mdm2 inhibitor (PDB code: 1T4E). Compound **70** is shown in yellow sticks, three water molecules as turquoise balls, the receptor amino acid side chains are labeled around the binding site. The hydrogen bonding network is shown in red dash line.

Since thienodiazepinediones are bioisosteric to benzodiazepindiones, we screened the compound library by our recently developed FP assay.[124] This robust FP assay uses fluorescent p53-like peptide and recombinant Mdm2 to measure the potency of p53-Mdm2 inhibitor. To our delight, **69a-7** and **69a-8** have shown inhibitor activities of p53-Mdm2 interaction through FP assay screening. Both inhibitors have dose dependent effect to compete with p53-like peptide.

The measured K_i values of Mdm2 with **69a-7** and **69a-8** were determined as 40 µM and 45 µM, respectively.

Compound **69a-7** was docked into the p53 binding site of Mdm2 in order to understand the possible binding mode of small molecular inhibitors (**Figure 38**). We were using the modeling/docking software MOLOC (Gerber, P.; www.moloc.ch).[111, 112] Compound **69a-6** also has weak interaction with Mdm2, which indicates that *p*-chlorobenzyl fragment might be crucial for the binding. This is consistent with other reported scaffolds, such as benzodiazepinedione **6**, as well as nutlins, chromenones and spirooxindoles.[28, 33, 260] Our recently described 3-finger pharmacophore model can be applied here and suggests that the compounds bind such that the *p*-chlorobenzyl group mimics Trp23, the cyclohexyl and the benzyl moiety mimics Leu26 and Phe19, respectively.[153] This is consistent with the docking model suggesting that the 4-chlorobenzyl group points deeply into tryptophan binding site.
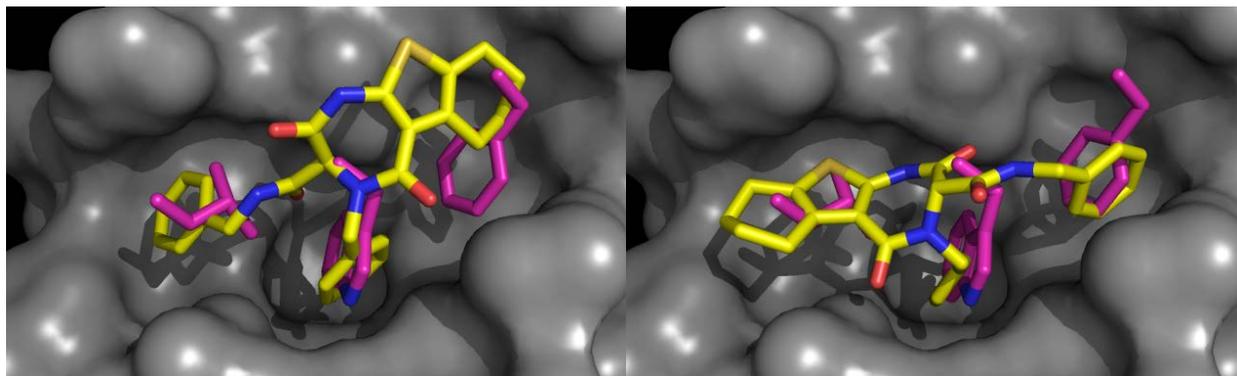
Figure 38. Compound **69a-7** (yellow sticks) docked into p53 binding site of Mdm2 (PDB code: 1YCR). Two low energy poses are shown (yellow sticks) as well as the side chains of the p53 hot spot FWL (pink sticks). In the first model the thiophene annulated cyclohexyl ring and the benzyl group mimics Phe19 and Leu26, respectively; whereas in the second model the cyclohexylthiophene fragment lays on top of the Leu26 binding site and the benzyl group points into the Phe19 binding site. Both poses suggest that there is not perfect shape and electrostatic complementarity which accounts for the moderate affinity for Mdm2.

Next, we utilized NMR to investigate the inhibitory behavior of compounds **69a-7** and **69a-8** towards the p53-Mdm2 interaction. For this purpose, we used the NMR-based AIDA that we developed recently.[117, 118] This NMR competition assay is performed on the Mdm2/p53 complex and the release of p53 from the complex is monitored as a function of the increased addition of an inhibitor. Therefore, AIDA-NMR indicates not only whether the compound is able to inhibit the protein-protein interaction in vitro, but also the dissociation constant (K_d) of the compound-Mdm2 interaction. The assay shows that the compounds are able to dissociate the Mdm2/p53 complex, and K_d values are 30 ± 20 μM and 10 ± 6 μM for compound **69a-7** and **69a-8**, respectively.

Furthermore, we used the HSQC NMR spectroscopy, which has been intensively used to monitor the biophysical properties of p53-Mdm2 inhibitors.[33, 261, 262] 2D HSQC spectra, showing binary titrations of ^{15}N-Mdm2 with compounds **69a-7** and **69a-8,** are shown in **Figure 39** and **Figure 40**, respectively. Although compound **69a-8** shows a stronger binding affinity, both inhibitors have similar modes of binding to Mdm2. The biggest chemical shift perturbations occur in proximity of the p53 binding site, as shown in **Figure 41**.
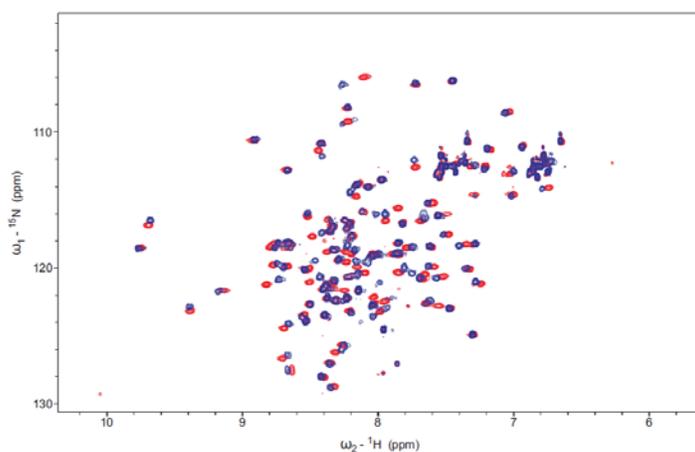
Figure 39. 2D ^{1}H-^{15}N HSQC spectrum of Mdm2 titrated with compound **69a-7**. (Blue: human Mdm2(1-118) mixed

molar with excess of compound **69a-7** (Mdm2:compound **69a-7** ratio 1:5); Red: the reference spectrum of free
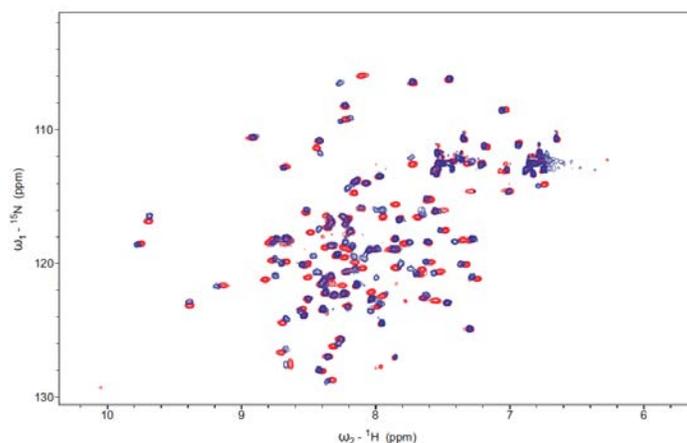
Mdm2)

Figure 40. 2D ^{1}H-^{15}N HSQC spectrum of Mdm2 titrated with compound **69a-8**. (Blue: human Mdm2(1-118) mixed

molar with excess of compound **69a-8** (Mdm2:compound **69a-8** ratio 1:5); Red: the reference spectrum of free

Mdm2)
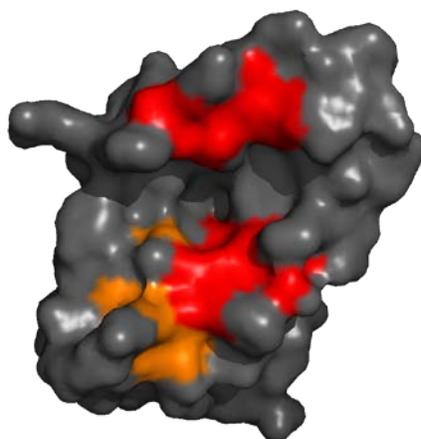
Figure 41. NMR mapping of binding site of compound **69a-8**. Atoms experiencing very large ($\Delta\delta > 0.08$ ppm) and

large ($\Delta\delta > 0.04$ ppm) differences in chemical shifts upon addition of molar excess of the compound are marked

red and orange, respectively. Assignment of the N-terminal domain of Mdm2 was published previously [33].

In summary, we have described the synthesis of the new scaffold TDZ using the unprecedented union of Gewald and Ugi MCRs and incorporating the UDC approach. The compounds accessible can be varied at four points in the molecular skeleton. The synthesis can be easily adapted for high throughput chemistry or medicinal chemistry for high-dimensional combinatorial libraries for screening purpose. The resulting compounds are in general drug-like by means of the Pfizer rules. Some compounds were shown to antagonize the p53-Mdm2 protein-protein interaction by two complementary screening techniques, and can provide a good starting point for further design and optimization. Moreover, we provide a useful program to generate the random virtual library for computational chemistry applications. Current efforts in our laboratories focus on the optimization of the potency of p53-Mdm2 inhibitors based on this interesting new class of compounds.

Materials and Methods

Methyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (66a): To a mixture of cyclohexanone (0.45 mol, 44.10 g), methyl cyanoacetate (0.3 mol, 28.77 g), sulfur (0.3 mol, 9.60 g), and 50 mL of ethanol, the solution of diethylamine (0.15 mol, 12.75 g) in 25 mL of ethanol was added dropwise with magnetic stirring. After the completion of the addition, the reaction mixture was kept stirring under room temperate overnight. The reaction flask was kept in the freezer for crystallization. Then the precipitate was collected by vacuum filtration, and washed by cold ethanol. After air dry overnight, 42.20 g of yellow solid were obtained (yield: 67%). HPLC/MS: t_R = 10.58 min; m/z = 212.1 $[M+H]^+$ ^{1}H NMR (600 MHz, CDCl$_3$): 1.73-1.78 (4H, m), 2.49-2.51 (2H, m), 2.67-2.69 (2H, m), 3.79 (3H, s, OMe), 5.93 (2H, br.s, NH$_2$). ^{13}C NMR (150 MHz, CDCl$_3$): 22.8, 23.3, 24.5, 26.9, 50.6, 105.6, 117.7, 132.4, 161.8, 166.5.

2-(Boc-amino)-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylic acid (67a): The mixture of

66a (10 mmol, 2.11 g), Boc$_2$O (12.5 mmol, 2.56 g), DMAP (1 mmol, 122 mg) in 25 mL of THF

was stirring under reflux overnight. The additional 0.5 g of Boc$_2$O was added, and stirring under

reflux overnight. After cooling to RT, the mixture was quenched by water, and extracted by

DCM. The organic layer was dried, and concentrated. The residue was dissolved in 20 mL of

ethanol, potassium hydroxide (2.24 g) and 20 mL of water was added. The mixture was reflux

for 5 hours. The reaction was quenched by water, the aqueous mixture was washed by ether. The

aqueous was the adjusted to pH = 6 with 1 M HCl aq. The product was collected by vacum

filtration as yellow solid (1.42 g, 48%). HPLC/MS: t_R = 7.93 min, *m/z* = 296.0 [M-H]$^-$. HRMS:

297.102519 (found); C$_{14}$H$_{19}$NO$_4$S, 297.1035 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 1.57 (9H, s),

1.79-1.82 (4H, m), 2.62-2.63 (2H, m), 2.82-2.83 (2H, m), 10.09 (1H, s). ^{13}C NMR (150 MHz,

CDCl$_3$): 22.7, 23.0, 24.2, 26.3, 28.2, 82.3, 109.2, 125.3, 131.7, 151.8, 170.7.

Methyl 2-aminothiophene-3-carboxylate (66b): Triethylamine (50 mmol, 5.0 mL) was added

dropwise to a mixture of 1,4-dithiane-2,5-diol (7.60 g, 50 mmol), methyl cyanoacetate (9.59 g,

100 mmol), and DMF (40 mL). The mixture was stirred at 45 ^{o}C for 30 min. After cooled to RT,

the reaction mixture was diluted with aqueous acetic acid (0.4 M, 200 mL). The mixture was

extracted with ether (4 x 40 mL), and the combined organic layer was washed with water (2 x 40

mL). After dried over sodium sulfate, the organic layer was filtered through silica gel pad. After

evaporation, 8.70 g of yellow solid were obtained (yield: 55%). HPLC/MS: t_R = 9.01 min; *m/z* =

158.2 [M+H]$^+$ ^{1}H NMR (600 MHz, CDCl$_3$): 3.83 (3H, s), 5.94 (2H, br.s), 6.20 (1H, d, *J* = 5.4

Hz), 6.98 (1H, d, *J* = 6.0 Hz). ^{13}C NMR (150 MHz, CDCl$_3$): 51.0, 106.9, 107.0, 125.8, 162.7,

165.8.

2-(Boc-amino)-3-thiophenecarboxylic acid (67b): The mixture of **66b** (20 mmol, 3.14 g), Boc$_2$O (25 mmol, 5.15 g), DMAP (2 mmol, 244 mg) in 30 mL of THF was stirring under reflux overnight. After evaporation of the solvent, the mixture was quenched by water, and extracted by DCM. The organic layer was dried, and concentrated. The residue was dissolved in 20 mL of ethanol, potassium hydroxide (4.5 g) and 20 mL of water was added. The mixture was reflux for 5 hours. The reaction was quenched by water, the aqueous mixture was washed by ether. The aqueous was the adjusted to pH = 6 with 1 M HCl aq. The product was collected by vacuum filtration and further purified by flash chromatography with ethyl acetate, 1.52 g of brown solid were obtained (yield: 31%). HPLC/MS: t_R = 10.37 min; m/z = 242.0 [M-H]$^-$. HRMS: 243.055945 (found); C$_{10}$H$_{13}$NO$_4$S, 243.05653 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 1.58 (9H, s), 6.71 (1H, d, J = 5.4 Hz), 7.23 (1H, d, J = 6.0 Hz), 9.88 (1H, br.s). ^{13}C NMR (150 MHz, d^6-DMSO): 28.2, 82.5, 112.5, 115.9, 125.0, 150.1, 151.8, 166.9.

Methyl 2-amino-5-phenylthiophene-3-carboxylate (66c): Phenyl-acetaldehyde (0.1 mol, 12.0 g), sulfur (0.1 mol, 3.20 g), methyl cyanoacetate (0.1 mol, 8.57 mL) were stirred at RT in absolute ethanol (50 mL). Triethylamine (0.1 mol, 10.0 mL) was added slowly. After the addition was completed, the mixture was heated at reflux for 1 hour. After cooled to RT, the precipitate was collected by vacuum filtration, and washed by cold ethanol. After air dry overnight, 14.6 g of yellow solid were obtained (yield: 63%). HPLC/MS: t_R = 11.01 min; m/z = 234.3 [M+H]$^+$ ^{1}H NMR (600 MHz, CDCl$_3$): 3.86 (3H, s), 6.02 (2H, br.s), 7.21-7.26 (2H, m), 7.33-7.46 (2H, m),7.45-7.46 (2H, m). ^{13}C NMR (150 MHz, CDCl$_3$): 51.1, 107.7, 124.7, 125.0, 126.6, 128.8, 133.9, 162.2, 165.8.

2-(Boc-amino)-5-phenylthiophene-3-carboxylic acid (67c): The mixture of **66c** (10 mmol, 2.33 g), Boc$_2$O (15 mmol, 3.10 g), DMAP (1 mmol, 122 mg) in 30 mL of THF was stirring

under reflux overnight. After cooling to RT, the mixture was quenched by water, and extracted by DCM. The organic layer was dried, and concentrated. The residue was dissolved in 20 mL of ethanol, potassium hydroxide (2.24 g) and 20 mL of water was added. The mixture was reflux for 1 hour. The reaction was quenched by water, the aqueous mixture was washed by ether. The aqueous was the adjusted to pH = 6 with 1 M HCl aq. The product was collected by vacum filtration as yellow solid (1.50 g, 47%). HPLC/MS: t_R = 11.79 min; m/z = 320.1 $[M+H]^+$. HRMS: 319.086854 (found); $C_{16}H_{17}NO_4S$, 319.08783 (calcd.). ^1H NMR (600 MHz, CDCl$_3$): 1.60 (9H, s), 7.29-7.31 (1H, m), 7.38-7.40 (2H, m), 7.45 (1H, s), 7.59-7.60 (2H, m), 9.90 (1H, s). ^{13}C NMR (150 MHz, CDCl$_3$): 28.2, 82.9, 110.9, 119.7, 125.3, 127.4, 129.0, 132.7, 133.5, 152.0, 152.3, 169.1.

Ethyl 2-[[(2-*tert*-butoxycarbonyl-amino-2,3,4,5,6,7-hexahydro-1-benzothien-3-yl) carbonyl] (benzyl)amino]-3-(*tert*-butyl-amino)-3-oxopropanoate (4a-1): The mixture of **67a** (74.3 mg, 0.25 mmol), benzylamine (0.25 mmol, 27.4 µL), ethyl glyoxylate (0.25 mmol, 49.6 µL), *tert*-butyl isocyanide (0.25 mmol, 28.3 µL) in 0.5 mL of methanol was stirring under RT for 2 days. The reaction was quenched by water, and extracted by DCM. The organic layer was washed by saturated potassium carbonate (aq), and dried over anhydrous sodium sulfate. After the evaporation of the solvent, the product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 5:1) as yellowish solid (93 mg, yield: 65%). HPLC/MS: t_R = 13.21 min; m/z = 572.2 $[M+H]^+$. HRMS: 571.272879 (found); $C_{30}H_{41}N_3O_6S$, 571.27161 (calcd.). ^1H NMR (600 MHz, CDCl$_3$): 1.24 (3H, t, J = 7.2 Hz), 1.39 (9H, s), 1.55 (9H, s), 1.76-1.82 (4H, m), 2.31 (1H, m), 2.63-2.67 (2H, m), 3.48 (1H, s), 4.09 (2H, m), 4.21 (1H, m), 4.42 (1H, d, J = 15.0 Hz), 4.99 (1H, d, J = 15.0 Hz), 7.27-7.32 (5H, m), 8.19 (1H, s), 9.63 (1H, s). ^{13}C NMR (150 MHz,

CDCl$_3$): 13.9, 22.6, 23.4, 23.8, 24.0, 28.3, 28.5, 52.1, 55.1, 61.4, 62.1, 80.7, 115.1, 126.6, 128.0, 128.4, 128.9, 129.4, 135.8, 137.2, 153.0, 164.6, 168.5, 169.0.

Method A for the synthesis of 69a

3,4,6,7,8,9-Hexahydro-3-(*N-tert*-butyl)-carboxamide-4-benzyl-1*H*-benzothieno-[2,3-e]-1,4-diazepine-2,5-dione (69a-1): The mixture of **67a** (74.3 mg, 0.25 mmol), benzylamine (0.25 mmol, 27.4 µL), ethyl glyoxylate (0.25 mmol, 49.6 µL), *tert*-butyl isocyanide (0.25 mmol, 28.3 µL) in 0.5 mL of methanol was stirring under RT for 2 days. The reaction was quenched by water, and extracted by DCM. The organic layer was washed by saturated potassium carbonate (aq), and dried over anhydrous sodium sulfate. After the evaporation of the solvent, the residue was treated by 0.5 mL of TFA, stirring under RT overnight. The reaction was added 10 mL of DCM, then neutralized by saturated potassium carbonate (aq). The mixture was extracted by DCM, the organic layer was combined and dried over anhydrous sodium sulfate. After the evaporation of the solvent, the residue was treated by triethylamine (50 µL) and TBD (10 mg) in 0.5 mL of THF, stirring overnight under 40 $^{\circ}$C. **69a-1** was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 3:1) as yellowish solid (35 mg, yield: 33% over three steps). HPLC/MS: t_R = 10.95 min, m/z = 426.2 [M+H]$^+$. HRMS: 425.176977 (found); C$_{23}$H$_{27}$N$_3$O$_3$S, 425.17731 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 0.86 (9H, s), 1.61 (1H, m), 1.79 (1H, m), 1.86 (1H, m), 1.94 (1H, m), 2.42 (1H, m), 2.60 (2H, m), 3.12 (1H, m), 3.99 (1H, d, J = 14.4 Hz), 4.62 (1H, s), 4.81 (1H, s), 5.63 (1H, d, J = 13.8 Hz), 7.39-7.43 (3H, m), 7.56-7.57 (2H, m). ^{13}C NMR (150 MHz, CDCl$_3$): 22.3, 23.0, 24.5, 26.1, 27.8, 51.3, 52.3, 67.1, 122.2, 128.7, 129.2, 129.6, 129.7, 134.1, 137.1, 104.8, 163.1, 163.7, 168.4.

3,4,6,7,8,9-Hexahydro-3-(*N-tert*-butyl)-carboxamide-4-phenethyl-1*H*-benzothieno-[2,3-e]-1,4-diazepine-2,5-dione (69a-2): The product was isolated by silica gel chromatography

(petroleum ether/ethyl acetate, 2:1) as yellowish solid (58 mg, yield: 53% over three steps). HPLC/MS: t_R = 10.93 min, m/z = 440.2 [M+H]$^+$. HRMS: 439.193180 (found); $C_{24}H_{29}N_3O_3S$, 439.19296 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 1.05 (9H, s), 1.61 (1H, m), 1.77 (1H, m), 1.83-1.90 (2H, m), 2.39 (1H, m), 2.58-2.59 (2H, m), 2.96-3.00 (1H, m), 3.01-3.07 (2H, m), 3.95-3.99 (2H, m), 4.60 (1H, s), 5.39 (1H, s), 7.22 (1H, m), 7.28-7.31 (4H, m). ^{13}C NMR (150 MHz, CDCl$_3$): 22.3, 23.0, 24.5, 25.8, 28.0, 34.1, 50.3, 51.8, 68.2, 123.2, 126.8, 128.7, 128.8, 129.9, 134.0, 137.6, 139.7, 163.5, 163.6, 168.9.

3,4,6,7,8,9-Hexahydro-3-(*N-tert*-butyl)-carboxamide-4-(4-chlorobenzyl)-1*H*-benzothieno-[2,3-e]-1,4-diazepine-2,5-dione (69a-3): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellowish solid (32 mg, yield: 28% over three steps). HPLC/MS: t_R = 11.32 min, m/z = 460.0 [M+H]$^+$. HRMS: 459.136130 (found); $C_{23}H_{26}ClN_3O_3S$, 459.13834 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 0.92 (9H, s), 1.61 (1H, m), 1.78 (1H, m), 1.86 (1H, m), 1.93 (1H, m), 2.40 (1H, m), 2.58-2.60 (2H, m), 3.09 (1H, s), 4.19 (1H, d, J = 14.4 Hz), 4.58 (1H, s), 4.86 (1H, s), 5.37 (1H, d, J = 13.2 Hz), 7.38 (1H, d, J = 7.8 Hz), 7.49 (1H, d, J = 8.4 Hz). ^{13}C NMR (150 MHz, CDCl$_3$): 22.3, 22.9, 24.5, 26.0, 27.9, 51.6, 51.9, 67.3, 122.3, 129.6, 129.9, 130.5, 134.1, 134.6, 135.4, 140.3, 163.2, 163.3, 168.4.

3,4,6,7,8,9-Hexahydro-3-(*N*-cyclopropylmethyl)-carboxamide-4-(4-chlorophenyl)-1*H*-benzothieno-[2,3-e]-1,4-diazepine-2,5-dione (69a-4): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 3:1) as yellowish solid (18 mg, yield: 16% over three steps). HPLC/MS: t_R = 11.10 min, m/z = 446.0 [M+H]$^+$. HRMS: 445.121614 (found); $C_{22}H_{24}ClN_3O_3S$, 445.12269 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 1.16 (9H, s), 1.61 (1H, m), 1.78 (1H, m), 1.86-1.88 (2H, m), 2.47 (1H, m), 2.61 (2H, m), 3.00 (1H, m), 4.88 (1H, s), 5.01 (1H, s), 7.33 (2H, d, J = 7.2 Hz), 7.39 (2H, d, J = 6.6 Hz).^{13}C NMR (150 MHz, CDCl$_3$): 22.3,

23.0, 24.6, 25.9, 28.2, 29.7, 52.3, 70.3, 123.4, 127.7, 129.7, 130.2, 133.3, 135.0, 141.3, 162.7, 168.4, 170.7.

3,4,6,7,8,9-Hexahydro-3-(*N*-cyclopropylmethyl)-carboxamide-4-benzyl-1*H*-benzothieno-[2,3-e]-1,4-diazepine-2,5-dione (69a-5): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellowish solid (27 mg, yield: 26% over three steps). HPLC/MS: t_R = 10.34 min, m/z = 424.3 [M+H]$^+$. HRMS: 423.161189 (found); $C_{23}H_{25}N_3O_3S$, 423.16166 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): -0.10 (2H, m), 0.32 (2H, m), 0.43 (1H, m), 1.76 (1H, m), 1.83 (1H, m), 1.89 (1H, m), 2.45 (1H, m), 2.51 (1H, m), 2.58-2.59 (2H, m), 2.77 (1H, m), 3.07 (1H, m), 4.22 (1H, d, J = 14.4 Hz), 4.63 (1H, s), 5.28 (1H, s), 5.45 (1H, d, J = 14.4 Hz), 7.35 (1H, m), 7.39-7.41 (2H, m), 7.53-7.54 (2H, m). ^{13}C NMR (150 MHz, CDCl$_3$): 3.29, 3.33, 10.1, 22.3, 23.0, 24.5, 25.9, 44.5, 52.4, 66.4, 122.1, 128.68, 128.74, 129.0, 129.49, 129.52, 134.3, 136.8, 140.5, 163.3, 164.2, 168.2.

3,4,6,7,8,9-Hexahydro-3-(*N*-mesityl)-carboxamide-4-(4-chlorobenzyl)-1*H*-benzothieno-[2,3-e]-1,4-diazepine-2,5-dione (69a-6): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellowish solid (15 mg, yield: 12% over three steps). HPLC/MS: t_R = 11.63 min, m/z = 522.2 [M+H]$^+$. HRMS: 521.153301 (found); $C_{28}H_{28}ClN_3O_3S$, 521.15399 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 1.26 (1H, m), 1.58 (6H, s), 1.77 (1H, m), 1.87-1.89 (2H, m), 2.17 (3H, m), 2.50-2.61 (3H, m), 3.08 (1H, m), 4.27 (1H, d, J = 14.4 Hz), 4.83 (1H, s), 5.35 (1H, d, J = 14.4 Hz), 6.58 (1H, s), 6.72 (2H, s), 7.32 (1H, d, J = 7.8 Hz), 7.45 (1H, d, J = 8.4 Hz). ^{13}C NMR (150 MHz, CDCl$_3$): 17.1, 20.8, 22.2, 22.9, 24.6, 26.1, 52.3, 67.0, 121.8, 128.9, 129.4, 129.6, 129.7, 130.5, 134.6, 134.7, 135.3, 137.4, 162.7, 163.5, 167.7.

3,4,6,7,8,9-Hexahydro-3-(*N*-benzyl)-carboxamide-4-(4-chlorobenzyl)-1*H*-benzothieno-[2,3-e]-1,4-diazepine-2,5-dione (69a-7): The product was isolated by silica gel chromatography

(petroleum ether/ethyl acetate, 2:1) as yellowish solid (20 mg, yield: 16% over three steps). HPLC/MS: t_R = 11.04 min, m/z = 494.0 [M+H]$^+$. HRMS: 493.122043 (found); $C_{26}H_{24}ClN_3O_3S$, 493.12269 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 1.60 (1H, m), 1.77 (1H, m), 1.87 (2H, m), 2.29 (1H, m), 2.59 (1H, m), 2.65 (1H, m), 3.01 (1H, m), 3.94 (1H, m), 4.07 (1H, m), 4.29 (1H, d, J = 14.4 Hz), 4.63 (1H, s), 5.22 (1H, d, J = 14.4 Hz), 5.47 (1H, s), 6.86 (2H, m), 7.20-7.21 (2H, m), 7.26-7.29 (3H, m), 7.34-7.36 (2H, m). ^{13}C NMR (150 MHz, CDCl$_3$): 22.2, 22.9, 24.6, 25.7, 44.1, 51.9, 66.6, 122.1, 127.9, 128.7, 129.5, 130.2, 134.6, 135.0, 136.6, 139.8, 163.3, 163.9, 167.6.

3,4,6,7,8,9-Hexahydro-3-(*N*-3,4-dichlorobenzyl)-carboxamide-4-(4-chlorobenzyl)-1*H*-benzothieno-[2,3-e]-1,4-diazepine-2,5-dione (69a-8): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 1:1) as yellowish solid (18 mg, yield: 16% over three steps). HPLC/MS: t_R = 11.72 min; m/z = 561.9 [M+H]$^+$. HRMS: 561.044289 (found); $C_{26}H_{22}Cl_3N_3O_3S$, 561.04475 (calcd.). ^{1}H NMR (600 MHz, CD$_3$OD): 1.35-1.36 (1H, m), 1.66-1.77 (3H, m), 2.12-2.15 (1H, m), 2.51-2.54 (2H, m), 2.87 (1H, m), 4.00 (1H , d, J = 14.4 Hz), 4.27 (1H , d, J = 14.4 Hz), 4.34 (1H , d, J = 15.0 Hz), 7.43 (1H , d, J = 15.0 Hz), 6.98 (1H, m), 7.31-7.33 (5H, m), 7.37 (1H, m). ^{13}C NMR (150 MHz, CD$_3$OD): 21.8, 22.6, 23.8, 25.0, 40.1, 42.0, 51.8, 121.5, 127.8, 128.4, 128.6, 129.6, 130.1, 130.2, 130.7, 131.6, 133.3, 133.9, 135.4, 139.4, 164.1, 165.8, 167.8.

Method B for the synthesis of 69b

Ethyl 2-[({2-[(*tert*-butoxycarbonyl)amino]thien-3-yl}carbonyl)(phenethyl)amino]-3-(*tert*-butyl-amino)-3-oxopropanoate (68b-1): The mixture of **67b** (74.3 mg, 0.25 mmol), 2-phenylethylamine (0.25 mmol, 31.4 µL), ethyl glyoxylate (0.25 mmol, 49.6 µL), *tert*-butyl isocyanide (0.25 mmol, 28.3 µL) in 0.5 mL of methanol was stirring under RT for 2 days. **68b-1** was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 5:1) as yellowish solid

(74 mg, yield: 56%). HPLC/MS: t_R = 12.57 min; m/z = 532.2 [M+H]$^+$. HRMS: 531.238491 (found); $C_{27}H_{37}N_3O_6S$, 531.24031 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 1.30 (3H, t, J = 7.2 Hz), 1.41 (9H, s), 1.50 (9H, s), 3.00 (2H, m), 3.81 (2H, m), 4.25-4.31 (2H, m), 4.49 (1H, s), 6.75 (1H, d, J = 5.4 Hz), 6.87 (1H, d, J = 6.0 Hz), 7.14-7.15 (2H, m), 7.20-7.23 (1H, m), 7.26-7.29 (2H, m), 7.84 (1H, br.s), 9.40 (1H, br.s). ^{13}C NMR (150 MHz, CDCl$_3$): 14.0, 28.2, 28.5, 35.4, 51.7, 62.3, 64.7, 73.0, 81.6, 114.1, 115.4, 122.7, 126.7, 128.6, 128.7, 137.8, 146.6, 152.3, 164.3, 168.4, 168.7.

3,4-Dihydro-3-(*N-tert*-butyl)-carboxamide-4-phenethyl-1*H*-thieno[2,3-e]-1,4-diazepine-2,5-dione (69b-1): The mixture of **68b-1** and 0.5 mL of DCM (10% TFA) was stirring under RT overnight. The reaction was added 10 mL of DCM, then neutralized by saturated potassium carbonate (aq). The mixture was extracted by DCM, the organic layer was combined and dried over anhydrous sodium sulfate. After the evaporation of the solvent, the residue was treated by triethylamine (50 µL) and TBD (10 mg) in 0.5 mL of THF, stirring overnight under 40 ^{o}C. **69b-1** was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 3:1) as yellowish solid (12 mg, yield: 22% over two steps). HPLC/MS: t_R = 9.79 min; m/z = 386.2 [M+H]$^+$. HRMS: 385.145394 (found); $C_{20}H_{23}N_3O_3S$, 385.14601 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 1.05 (9H, s), 2.96-3.11 (2H, m), 3.92-4.05 (2H, m), 4.67 (1H, s), 5.35 (1H, br.s), 6.85 (1H, d, J = 5.4 Hz), 7.21-7.24 (2H, m), 7.28-7.33 (4H, m). ^{13}C NMR (150 MHz, CDCl$_3$): 28.1, 34.3, 51.0, 52.0, 68.4, 118.2, 124.6, 126.8, 127.6, 128.77, 128.79, 137.5, 163.5, 168.5.

Ethyl 2-[({2-[(*tert*-butoxycarbonyl)amino]thien-3-yl}carbonyl)(2-(thiophen-3-yl)ethyl) amino]-3-(*tert*-butyl-amino)-3-oxopropanoate (68b-2): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 5:1) as yellowish solid (60 mg, yield: 45%). HPLC/MS: t_R = 12.44 min; m/z = 538.2 [M+H]$^+$. HRMS: 537.194766 (found); $C_{25}H_{35}N_3O_6S_2$,

537.19673 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 1.30 (3H, t, J = 7.2 Hz), 1.41 (9H, s), 1.51 (9H, s), 3.24 (2H, m), 3.82-3.87 (2H, m), 4.25-4.32 (2H, m), 4.46 (1H, s), 6.75 (1H, m), 6.82 (1H, m), 6.86 (1H, m), 6.92 (1H, m), 7.15 (1H, m), 7.85 (1H, br.s), 9.44 (1H, br.s). ^{13}C NMR (150 MHz, CDCl$_3$): 14.0, 28.2, 28.5, 29.5, 51.7, 62.3, 64.6, 72.9, 81.6, 113.9, 115.5, 122.6, 124.1, 125.5, 127.1, 139.9, 146.9, 152.3, 164.1, 168.3, 168.8.

3,4-Dihydro-3-(*N-tert*-butyl)-carboxamide-4-(2-(thiophen-3-yl)ethyl)-1*H*-thieno[2,3-e]-1,4-diazepine-2,5-dione (69b-2): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellowish solid (19 mg, yield: 43% over two steps). HPLC/MS: t_R = 9.64 min; m/z = 392.1 [M+H]$^+$. HRMS: 391.101427 (found); C$_{18}$H$_{21}$N$_3$O$_3$S$_2$, 391.10243 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 1.04 (9H, s), 3.21-3.31 (2H, m), 3.99-4.02 (2H, m), 4.68 (1H, s), 5.40 (1H, br.s), 6.83 (1H, m), 6.93 (1H, m), 6.96 (1H, m), 7.18 (1H, m), 7.20 (1H, m). ^{13}C NMR (150 MHz, CDCl$_3$): 28.0, 28.5, 51.0, 52.0, 68.5, 118.1, 124.4, 125.8, 127.2, 127.5, 140.0, 163.5, 163.7, 168.6.

Ethyl 2-[({2-[(*tert*-butoxycarbonyl)amino]thien-3-yl}carbonyl)(phenyl)amino]-3-(cyclohexyl-amino)-3-oxopropanoate (68b-3): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 5:1) as yellowish solid (70 mg, yield: 52%). HPLC/MS: t_R = 12.75 min; m/z = 544.0 [M+H]$^+$. HRMS: 543.238512 (found); C$_{28}$H$_{37}$N$_3$O$_6$S, 543.24031 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 1.27 (2H, m), 1.30 (3H, t, J = 7.2 Hz), 1.37-1.38 (1H, m), 1.39-1.41 (2H, m), 1.52 (9H, s), 1.58 (1H, m), 1.68-1.70 (2H, m), 1.93 (2H, m), 3.83 (1H, s), 4.21-4.24 (2H, m), 4.28-4.29 (1H, m), 4.81 (1H, d, J = 17.4 Hz), 5.08 (1H, d, J = 16.8 Hz), 6.60 (1H, d, J = 6.0 Hz), 6.87 (1H, d, J = 5.4 Hz), 7.32-7.35 (1H, m), 7.39-7.42 (2H, m), 7.51-7.52 (2H, m), 7.92 (1H, br.s), 9.79 (1H, br.s). ^{13}C NMR (150 MHz, CDCl$_3$): 14.0, 24.5,

25.6, 28.2, 32.4, 32.5, 48.4, 62.2, 63.4, 81.8, 112.9, 115.0, 122.8, 127.2, 127.9, 128.9, 135.9, 149.4, 152.3, 164.3, 168.3, 169.0.

3,4-Dihydro-3-(*N*-cyclohexyl)-carboxamide-4-benzyl-1*H*-thieno[2,3-e]-1,4-diazepine-2,5-dione (69b-3): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 3:1) as yellowish solid (10 mg, yield: 20% over two steps). HPLC/MS: t_R = 10.01 min; m/z = 398.1 [M+H]$^+$. HRMS: 397.145096 (found); $C_{21}H_{23}N_3O_3S$, 397.14601 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 0.49 (1H, m), 0.59 (1H, m), 0.98-1.02 (1H, m), 1.09-1.11 (2H, m), 1.24-1.27 (2H, m), 1.46 (3H, m), 3.25 (1H, m), 4.07 (1H, d, J = 13.8 Hz), 4.72 (1H, s), 4.84 (1H, br.s), 5.59 (1H, d, J = 14.4 Hz), 6.84 (1H, d, J = 5.4 Hz), 7.20 (1H, d, J = 5.4 Hz), 7.40-7.44 (3H, m), 7.59-7.60 (2H, m). ^{13}C NMR (150 MHz, CDCl$_3$): 24.4, 24.5, 25.2, 31.9, 32.2, 48.1, 52.8, 66.5, 118.0, 123.6, 127.8, 128.9, 129.4, 129.7, 136.7, 163.1, 163.3, 167.7.

Ethyl 2-[({2-[(*tert*-butoxycarbonyl)amino]thien-3-yl}carbonyl)(3,4-dimethoxy phenethyl) amino]-3-(cyclopropylmethyl-amino)-3-oxopropanoate (68b-4): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 5:1) as yellowish solid (53 mg, yield: 36%). HPLC/MS: t_R = 11.70 min; m/z = 590.2 [M+H]$^+$. HRMS: 589.248129 (found); $C_{29}H_{39}N_3O_8S$, 589.24579 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 0.24-0.26 (2H, m), 0.53-0.54 (2H, m), 1.01 (1H, m), 1.30 (3H, t, J = 7.2 Hz), 1.51 (9H, s), 2.95-2.99 (2H, m), 3.17-3.26 (2H, m), 3.84 (3H, s), 3.85 (3H, s), 4.25-4.34 (2H, m), 4.56 (1H, s), 6.65 (1H, m), 6.68-6.70 (1H, m), 6.75-6.78 (2H, m), 6.92 (1H, d, J = 5.4 Hz), 7.98 (1H, br.s), 9.40 (1H, br.s). ^{13}C NMR (150 MHz, CDCl$_3$): 3.3, 3.4, 10.5, 14.0, 28.2, 34.9, 44.6, 55.86, 55.90, 62.3, 64.2, 81.9, 111.3, 111.8, 113.8, 115.3, 120.6, 122.8, 130.2, 147.5, 147.8, 149.0, 152.3, 165.4, 168.4.

3,4-Dihydro-3-(*N*-cyclopropylmethyl)-carboxamide-4-(3,4-dimethoxyphenethyl)-1*H*-thieno[2,3-e]-1,4-diazepine-2,5-dione (69b-4): The product was isolated by silica gel

chromatography (petroleum ether/ethyl acetate, 3:1) as yellowish solid (10 mg, yield: 38% over two steps). HPLC/MS: t_R = 8.98 min; m/z = 444.0 [M+H]$^+$. HRMS: 443.151569 (found); $C_{22}H_{25}N_3O_5S$, 443.15149 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): -0.03 (2H, m), 0.35 (2H, m), 0.54 (1H, m), 2.65 (1H, m), 2.94-3.06 (3H, m), 3.85 (3H, s), 3.88 (3H, s), 3.96-4.11 (2H, m), 4.69 (1H, s), 5.60 (1H, br.s), 6.80-6.85 (4H, m), 7.17 (1H, d, J = 5.4 Hz). ^{13}C NMR (150 MHz, CDCl$_3$): 3.35, 3.41, 10.4, 33.6, 44.7, 55.88, 55.92, 111.4, 111.9, 113.9, 118.2, 120.8, 127.7, 129.7, 141.7, 147.8, 149.0, 163.4, 163.9, 167.6.

Method C for the synthesis of 69c

Ethyl 2-[({2-[(*tert*-butoxycarbonyl)amino]-5-phenylthien-3-yl}carbonyl) (cyclopropyl methyl)amino]-3-(*tert*-butyl-amino)-3-oxopropanoate (68c-1): The mixture of **67c** (0.2 mmol, 63.8 mg), cyclopropyl methamine (0.2 mmol, 17.3 µL), ethyl glyoxylate (0.2 mmol, 39.7 µL), *tert*-butyl isocyanide (0.2 mmol, 22.6 µL) in 0.5 mL of methanol was stirring under RT for 2 days. **68c-1** was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 5:1) as yellowish solid (46 mg, yield: 41%). HPLC/MS: t_R = 13.10 min; m/z = 558.2 [M+H]$^+$. HRMS: 557.255949 (found); $C_{29}H_{39}N_3O_6S$, 557.25596 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 0.19-0.28 (2H, m), 0.60-0.65 (2H, m), 1.10-1.12 (1H, m), 1.31 (3H, t, J = 7.2 Hz), 1.41 (9H, s), 1.53 (9H, s), 3.38-3.42 (1H, m), 3.69-3.72 (1H, m), 4.29 (2H, q, J = 7.2 Hz), 4.65 (1H, s), 7.19 (1H, s), 7.25-7.27 (1H, m), 7.36-7.38 (2H, m), 7.56-7.57 (2H, m), 8.06 (1H, s), 9.53 (1H, s). ^{13}C NMR (150 MHz, CDCl$_3$): 3.4, 4.5, 10.0, 14.0, 28.2, 28.6, 51.6, 62.2, 63.4, 81.8, 115.2, 118.7, 125.2, 127.2, 129.0, 133.3, 133.9, 145.6, 152.4, 164.7, 167.8, 169.1.

7-Phenyl-3,4-dihydro-3-(*N*-cyclopropylmethyl)-carboxamide-4-cyclopropylmethyl-1*H*-thieno[2,3-e]-1,4-diazepine-2,5-dione (69c-1): The mixture of **68c-1** and 0.5 mL of DCM (10% TFA) was stirring under RT overnight. After the evaporation of the solvent, the residue was

treated by triethylamine (100 µL) and TBD (10 mg) in 0.5 mL of THF, stirring overnight under 40 °C. **69c-1** was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 3:1) as yellowish solid (12 mg, yield: 35% over two steps). HPLC/MS: t_R = 10.45 min; m/z = 412.2 [M+H]$^+$. HRMS: 411.161429 (found); $C_{22}H_{25}N_3O_3S$, 411.16166 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 0.47 (1H, m), 0.54 (1H, m), 0.62 (1H, m), 0.67 (1H, m), 1.15 (9H, s), 1.25 (1H, m), 3.33 (1H, m), 3.90 (1H, m), 4.81 (1H, s), 5.91 (1H, s), 7.29 (1H, m), 7.35-7.38 (2H, m), 7.43 (1H, s), 7.48-7.49 (2H, m). ^{13}C NMR (150 MHz, CDCl$_3$): 3.7, 4.4, 10.2, 28.2, 52.1, 53.7, 67.9, 122.6, 124.9, 125.5, 128.1, 129.1, 132.8, 136.7, 141.7, 163.2, 164.1, 168.2.

Ethyl 2-[({2-[(*tert*-butoxycarbonyl)amino]-5-phenylthien-3-yl}carbonyl)(2-methoxy-ethyl)amino]-3-(*tert*-butyl-amino)-3-oxopropanoate (68c-2): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 5:1) as yellow oil (55 mg, yield: 49%). HPLC/MS: t_R = 12.81 min; m/z = 562.1 [M+H]$^+$. HRMS: 561.250182 (found); $C_{28}H_{39}N_3O_7S$, 561.25087 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 1.31 (3H, t, J = 7.2 Hz), 1.41 (9H, s), 1.52 (9H, s), 3.36 (3H, s), 3.65-3.71 (2H, m), 3.81 (2H, m), 4.27 (2H, m), 4.72 (1H, s), 7.21 (1H, s), 7.23-7.26 (1H, m), 7.34-7.36 (2H, m), 7.53-7.55 (2H, m), 7.93 (1H, br.s), 9.54 (1H, br.s). ^{13}C NMR (150 MHz, CDCl$_3$): 14.1, 28.2, 28.5, 51.6, 59.0, 62.1, 64.5, 71.2, 81.8, 115.1, 119.0, 125.1, 127.2, 128.9, 133.2, 133.9, 145.6, 152.4, 164.3, 168.2, 168.9.

7-Phenyl-3,4-dihydro-3-(*N*-*tert*-butyl)-carboxamide-4-(2-methoxyethyl)-1*H*-thieno [2,3-e]-1,4-diazepine-2,5-dione (69c-2): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 3:1) as yellowish solid (11 mg, yield: 27% over two steps). HPLC/MS: t_R = 10.12 min; m/z = 416.0 [M+H]$^+$. HRMS: 415.155023 (found); $C_{21}H_{25}N_3O_4S$, 415.15658 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 1.11 (9H, s), 3.20-3.24 (1H, m), 3.43 (3H, s), 3.63-3.66 (1H, m), 3.89-3.91 (1H, m), 4.53-4.57 (1H, m), 4.71 (1H, s), 6.91 (1H, s), 7.28-7.29

(2H, m), 7.33-7.35 (2H, m), 7.38 (1H, s), 7.45-7.47 (2H, m). ^{13}C NMR (150 MHz, CDCl$_3$): 28.2, 48.9, 51.4, 58.7, 66.4, 68.4, 122.5, 124.6, 125.5, 127.9, 129.0, 133.1, 136.2, 163.8, 164.7, 168.7.

Ethyl 2-[({2-[(*tert*-butoxycarbonyl)amino]-5-phenylthien-3-yl}carbonyl)(phenethyl) amino]-3-(*tert*-butyl-amino)-3-oxopropanoate (68c-3): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 5:1) as yellowish solid (63 mg, yield: 52%). HPLC/MS: t_R = 13.43 min; m/z = 608.1 [M+H]$^+$. HRMS: 607.270091 (found); C$_{33}$H$_{41}$N$_3$O$_6$S, 607.27161 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 1.34 (3H, t, J = 7.2 Hz), 1.44 (9H, s), 1.53 (9H, s), 3.06 (2H, m), 3.89 (2H, m), 4.28-4.34 (2H, m), 4.53 (1H, s), 7.10 (1H, s), 7.19-7.20 (2H, m), 7.22-7.29 (4H, m), 7.35-7.37 (2H, m), 7.50-7.51 (2H, m), 7.88 (1H, br.s), 9.48 (1H, br.s).^{13}C NMR (150 MHz, CDCl$_3$): 14.1, 28.2, 28.6, 35.4, 51.7, 62.4, 64.7, 81.9, 114.9, 118.4, 125.2, 126.8, 127.2, 128.7, 128.8, 128.9, 133.3, 133.8, 137.9, 145.8, 152.3, 168.2, 168.8.

7-Phenyl-3,4-dihydro-3-(*N-tert*-butyl)-carboxamide-4-phenethyl-1*H*-thieno[2,3-e]-1,4-diazepine-2,5-dione (69c-3): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 3:1) as yellowish solid (18 mg, yield: 38% over two steps). HPLC/MS: t_R = 10.89 min; m/z = 462.0 [M+H]$^+$. HRMS: 461.175973 (found); C$_{26}$H$_{27}$N$_3$O$_3$S, 461.17731 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 1.04 (9H, s), 2.95-3.10 (2H, m), 3.92-4.03 (2H, m), 4,71 (1H, s), 5.45 (1H, br.s), 7.19-7.21 (1H, m), 7.26-7.28 (5H, m), 7.30-7.32 (2H, m), 7.40-7.44 (3H, m). ^{13}C NMR (150 MHz, CDCl$_3$): 28.1, 34.3, 50.9, 52.1, 68.5, 122.5, 125.1, 125.5, 126.8, 128.0, 128.8, 129.0, 132.9, 137.5, 163.6, 164.0, 168.8.

Ethyl 2-[({2-[(*tert*-butoxycarbonyl)amino]-5-phenylthien-3-yl}carbonyl)(2-methoxy-ethyl)amino]-3-(cyclopropylmethyl-amino)-3-oxopropanoate (68c-4): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 5:1) as yellowish solid (42 mg, yield: 38%). HPLC/MS: t_R = 12.47 min; m/z = 560.1 [M+H]$^+$. HRMS: 559.236369 (found);

C$_{28}$H$_{37}$N$_3$O$_7$S, 559.23522 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 0.27-0.29 (2H, m), 0.54-0.55 (2H, m), 1.04 (1H, m), 1.32 (3H, t, J = 7.2 Hz), 1.54 (9H, s), 3.22-3.28 (2H, m), 3.39 (3H, s), 3.70-3.75 (2H, m), 3.83-3.92 (2H, m), 4.27-4.32 (2H, m), 4.84 (1H, s), 7.24-7.28 (2H, m), 7.35-7.38 (2H, m), 7.54-7.55 (2H, m), 8.18 (1H, s), 9.51 (1H, s). ^{13}C NMR (150 MHz, CDCl$_3$): 3.37, 3.40, 10.5, 14.1, 28.2, 39.1, 44.5, 59.1, 62.2, 71.2, 82.0, 114.7, 119.1, 125.2, 127.2, 128.4, 128.9, 133.1, 133.9, 152.3, 165.3, 168.3, 168.7.

7-Phenyl-3,4-dihydro-3-(*N*-cyclopropylmethyl)-carboxamide-4-(2-methoxyethyl)-1*H*-thieno[2,3-e]-1,4-diazepine-2,5-dione (69c-4): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 1:1) as yellowish solid (9 mg, yield: 29% over two steps). HPLC/MS: t_R = 9.69 min; m/z = 414.3 [M+H]$^+$. HRMS: 413.140096 (found); C$_{21}$H$_{23}$N$_3$O$_4$S, 413.14093 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 0.01-0.05 (2H, m), 0.30-0.38 (2H, m), 0.69-0.71 (1H, m), 2.84-2.86 (1H, m), 2.94-2.98 (1H, m), 3.29-3.32 (1H, m), 3.45 (3H, s), 3.65-3.67 (1H, m), 3.94 (1H, m), 4.51-4.54 (1H, m), 4.80 (1H, s), 7.24-7.27 (1H, m), 7.30-7.32 (2H, m), 7.38 (2H, m), 7.43-7.45 (2H, m). ^{13}C NMR (150 MHz, CDCl$_3$): 3.37, 3.39, 10.5, 44.6, 49.1, 58.7, 67.9, 69.5, 122.4, 124.4, 125.4, 127.9, 129.0, 132.9, 136.2, 142.8, 163.8, 165.4, 168.3.

Ethyl 2-[({2-[(*tert*-butoxycarbonyl)amino]-5-phenylthien-3-yl}carbonyl)(2-methoxy-ethyl)amino]-3-(cyclohexyl-amino)-3-oxopropanoate (68c-5): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 5:1) as yellowish solid (82 mg, yield: 70%). HPLC/MS: t_R = 13.04 min; m/z = 588.3 [M+H]$^+$. HRMS: 587.265023 (found); C$_{30}$H$_{41}$N$_3$O$_7$S, 587.26652 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 1.21-1.25 (3H, m), 1.29 (3H, t, J = 7.2 Hz), 1.35-1.42 (2H, m), 1.52 (9H, s), 1.58-1.60 (2H, m), 1.71 (2H, m), 1.94-1.96 (2H, m), 3.36 (3H, s), 3.67 (1H, m), 3.73 (1H, m), 3.80-3.82 (3H, m), 4.27 (2H, m), 4.80 (1H, s), 7.21-

7.25 (2H, m), 7.33-7.35 (2H, m), 7.52-7.53 (2H, m), 7.97 (1H, d, J = 7.2 Hz), 9.47 (1H, s). ^{13}C NMR (150 MHz, CDCl$_3$): 14.1, 24.6, 25.6, 28.2, 32.5, 32.6, 48.6, 59.0, 62.1, 64.3, 71.0, 81.9, 114.9, 119.0, 125.1, 127.2, 128.9, 133.2, 133.9, 146.2, 152.3, 164.2, 168.4, 168.5.

7-Phenyl-3,4-dihydro-3-(*N*-cyclohexyl)-carboxamide-4-(2-methoxyethyl)-1*H*-thieno [2,3-e]-1,4-diazepine-2,5-dione (69c-5): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellowish solid (12 mg, yield: 19% over two steps). HPLC/MS: t_R = 10.44 min; m/z = 442.1 [M+H]$^+$. HRMS: 441.171348 (found); C$_{23}$H$_{27}$N$_3$O$_4$S, 441.17223 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 0.85-0.87 (1H, m), 1.06-1.16 (3H, m), 1.27-1.28 (1H, m), 1.44-1.51 (2H, m), 1.64-1.66 (1H, m), 1.76-1.77 (1H, m), 3.27-3.28 (1H, m), 3.45 (3H, s), 3.54-3.56 (1H, m), 3.64-3.66 (1H, m), 3.96 (1H, m), 4.54-4.57 (1H, m), 4.76 (1H, s), 7.07 (1H, d, J = 7.2 Hz), 7.28-7.30 (1H, m), 7.33-7.36 (2H, m), 7.45-7.46 (2H, m). ^{13}C NMR (150 MHz, CDCl$_3$): 24.5, 24.7, 25.4, 32.4, 32.7, 48.3, 49.2, 58.7, 67.9, 69.5, 122.6, 124.5, 125.5, 127.9, 129.0, 133.0, 136.3, 151.6, 163.7, 164.5, 168.3.

Ethyl 2-[({2-[(*tert*-butoxycarbonyl)amino]-5-phenylthien-3-yl}carbonyl)(2-cyclopropylmethyl)amino]-3-(cyclohexyl-amino)-3-oxopropanoate (68c-6): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 5:1) as yellowish solid (70 mg, yield: 60%). HPLC/MS: t_R = 13.36 min; m/z = 584.2 [M+H]$^+$. HRMS: 583.271707 (found); C$_{31}$H$_{41}$N$_3$O$_6$S, 583.27161 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 0.19-0.24 (2H, m), 0.62-0.63 (2H, m), 1.11 (1H, m), 1.25 (1H, m), 1.29 (3H, t, J = 7.2 Hz), 1.31-1.34 (2H, m), 1.38-1.42 (2H, m), 1.52 (9H, s), 1.55-1.59 (1H, m), 1.70 (2H, m), 1.93 (2H, m), 3.42-3.44 (1H, m), 3.67-3.70 (1H, m), 3.81-3.87 (1H, m), 4.28 (2H, q, J = 7.2 Hz), 4.74 (1H, s), 7.19 (1H, s), 7.24-7.27 (1H, m), 7.35-7.37 (2H, m), 7.54-7.56 (2H, m), 8.05 (1H, d, J = 7.2 Hz), 9.46 (1H, br.s). ^{13}C NMR

(150 MHz, CDCl$_3$): 3.6, 4.5, 10.0, 14.1, 24.5, 25.6, 28.2, 32.4, 32.6, 48.5, 55.5, 62.2, 63.2, 82.0, 115.0, 118.8, 125.2, 127.3, 129.0, 133.3, 133.9, 146.3, 152.3, 165.0, 168.0, 168.7.

7-Phenyl-3,4-dihydro-3-(*N*-cyclohexyl)-carboxamide-4-cyclopropylmethyl-1*H*-thieno[2,3-e]-1,4-diazepine-2,5-dione (69c-6): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellowish solid (13 mg, yield: 25% over two steps). HPLC/MS: t_R = 10.67 min; *m/z* = 438.2 [M+H]$^+$. HRMS: 437.176794 (found); C$_{24}$H$_{27}$N$_3$O$_3$S, 437.17731 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 0.46-0.53 (2H, m), 0.60-0.63 (2H, m), 0.87-0.91 (1H, m), 1.05-1.10 (2H, m), 1.15-1.32 (4H, m), 1.46-1.50 (2H, m), 1.61-1.63 (1H, m), 1.72-1.74 (1H, m), 3.31-3.34 (1H, m), 3.60-3.62 (1H, m), 3.88-3.92 (1H, m), 4.86 (1H, s), 6.02 (1H, d, *J* = 7.2 Hz), 7.29 (1H, m), 7.33-7.35 (2H, m), 7.39 (1H, s), 7.45-7.46 (2H, m). ^{13}C NMR (150 MHz, CDCl$_3$): 3.7, 4.4, 10.3, 24.5, 24.7, 25.3, 32.5, 32.6, 48.5, 53.8, 67.6, 122.8, 124.4, 125.4, 127.9, 129.0, 133.0, 136.2, 163.4, 164.3, 168.2.

Virtual Library Generation

The virtual library of Gewald-UDC thienodiazepinediones was created using the Reactor software as described in the manual.[110] As starting materials, we used commercially available 2-aminothiophenes (N = 120), 349 primary amines and 322 isocyanides. The isocyanides are either commercially available or can be synthesized in 1-2 steps using the classical Hoffmann or Ugi syntheses.[263] Consequently the theoretical chemical space of this virtual library is 120 x 349 x 322 = 13,485,360 (not including stereoisomers), which is almost unmanageable when calculating the physicochemical properties. As the father of modern MCR chemistry, Ivar Ugi pointed out in 1971 "if, for example, 40 each of the different components are reacted with one another, the result is 40^4 = 2,560,000 reaction products, which is quite a high figure considering that it is the same order of magnitude as the total number of chemical compounds described to date".[263]

Nowadays more than 1,000 isocyanides are commercially available, thus the U-4CR space increased to $1,000^4 = 10^{11}$ compounds which is out of reach of every current super computers. Thus, in order to investigate such large chemical spaces the program *RandReactor* was written to provide smaller random sublibraries of such very large chemical spaces.

3.3.2 Design of anchor-directed thienodiazepine scaffold*

* Adapted with permission from John Wiley and Sons: 2757771131537

Over 40 medications highlight 1,4-benzodiazepine as a classic privileged structure with a broad range of therapeutic treatment.[264] Since the discovery of benzodiazepine family, many synthetic derivatives with a wide pharmacological spectrum have been extensively developed.[265, 266] For example, farnesyltransferase (FTase) inhibitor BMS-214662 has undergone evaluation as an anti-cancer drug in phase I and II clinical trials.[267] Moreover, 1,4-benzodiazepines may act as mimetics of peptide secondary structures such as α-helix and β-turn, due to their unique structural motifs and physicochemical properties.[258, 268-270] It is noteworthy that 1,4-benzodiazepine-2,5-diones (BDZ) as a sub-family of benzodiazepine scaffolds have been synthesized as cyclic peptide structures with diversely substituted moieties.[271-273] Recently, hybrid peptidomimetic BDZs have been investigated during the lead generation process, such as tripeptide compounds **71-73** (**Figure 42**).[274-278] Therefore, the development of new synthetic scaffolds based on 1,4-diazepines has attracted considerable attention in the design of biologically active compounds.[279, 280]

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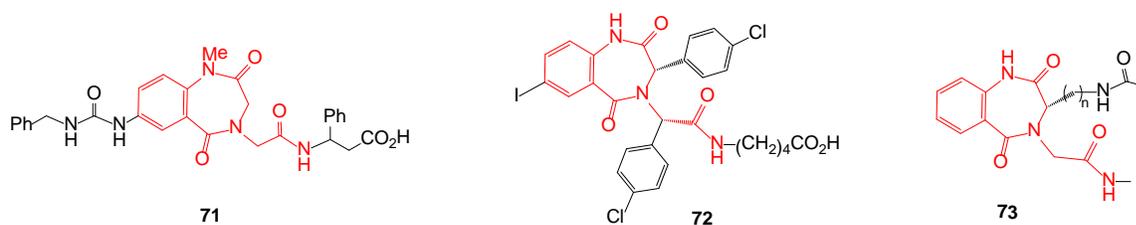
Figure 42. Hybrid peptidomimetic 1,4-benzodiazepine-2,5-diones

The chemistry developed based on 1,4-benzodiazepine scaffolds has contributed to the emergence of thiophene as a useful isostere of a benzene ring.[281] Bioisosterism successfully led to the discovery of a series of 1,4-thienodiazepine drugs (such as olanzapine, clotiazepam, and brotizolam), which are heterocyclic compounds containing a 1,4-diazepine ring fused to a thiophene ring. It's of great interest to develop the synthesis of new 1,4-thienodiazepine scaffolds, which are much less investigated compared to 1,4-benzodiazepines. Recently, compound **74** has been identified as an inhibitor of MAPKAP Kinase-2 (MK-2).[282] The co-crystal structure of compound **74** and MK-2 complex reveals the unique properties of a 1,4-thienodiazepine scaffold (**Figure 43**).[282] However, chemical space and the structural biology of 1,4-thienodiazepines is still largely unexplored. We are particularly interested in the synthesis of new TDZ scaffolds, which have been not investigated as potential pharmacophore so far.
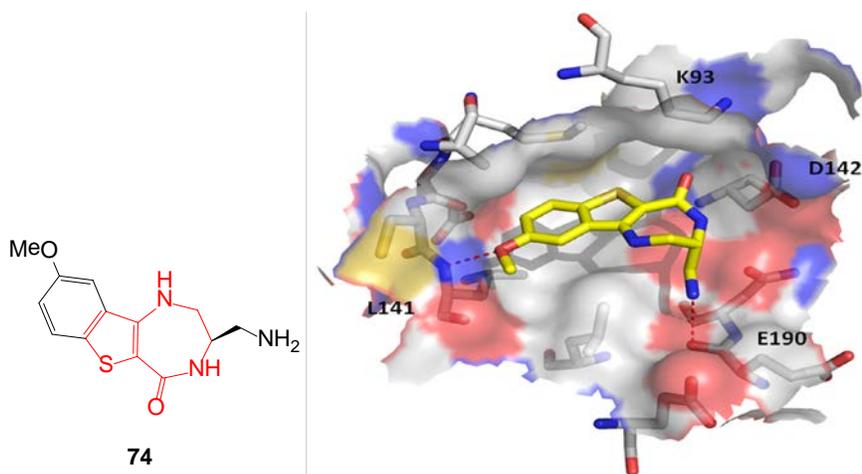
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MCR chemistry is particularly useful for the fast and efficient discovery of diverse scaffolds.[107] IMCRs provide powerful tools for producing diverse arrays of compounds with high atom economy.[242] In search of a novel synthetic route for the construction of TDZ scaffolds, 2-aminothiophene has been considered as the most appropriate precursor. Gewald-3CR is a unique method using elemental sulfur to yield 2-aminothiophenes **75**, which builds a platform for the synthesis of new thiophene scaffolds.[209, 210] We found that Gewald 2-aminothiophene is clearly an isostere of anthranilic acids.[214] Ugi-4CR is known to be one of the most versatile tools for the construction of α-aminoacylcarbonamides and related backbones.[126] For example, orthogonally protected anthranilic acids were used as a key synthon for the synthesis of 1,4-benzodiazepin-2,5-diones (BDZs) via the UDC approach.[244, 246, 283, 284] In our recent study, we developed UDC strategy using 2-aminothiophene derivatives **76** and ethyl glyoxalate to access TDZ derivatives as potential p53-Mdm2 inhibitors.[125] Herein, we utilized the UDC strategy (**Figure 44**) to synthesize another new scaffold of TDZs starting from 2-aminothiophenes, which were prepared by Gewald reactions.
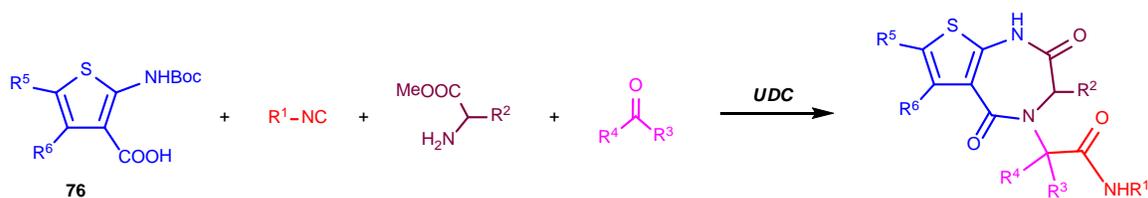
Figure 44. UDC approach for the synthesis of 1,4-thienodiazepine-2,5-dione scaffolds (R^1, R^2, R^3, R^4, R^5, R^6: points of diversity)

Synthesis of 1,4-thienodiazepine-2,5-dione scaffold

Since 2-aminothiophenes are optimal starting materials for the synthesis of thienodiazepine backbone, we utilized the versatile Gewald MCRs to prepare compounds **76a-c**. We recently developed a general synthetic protocol to synthesize Boc protected thiophene carboxylic acids **6a-c** (**Scheme 26**).[125] In the first step, 2-aminothiophenes **75a-c** were obtained by the Gewald reaction of cyclohexanone, 1,4-dithiane-2,5-diol, phenylacetaldehyde, respectively. In the second step, *N*-Boc thiophene carboxylic acids **76** were prepared by Boc protection of **75** and following hydrolysis transformation. Hence, we intended to employ the bifunctional orthogonally protected intermediates **76** and amino acid derived methyl esters for the synthesis of new TDZ scaffold via UDC approach.

75a, R^5, R^6 = (⁻CH$_2$⁻)$_4$
75b, R^5, R^6 = H
75c, R^5 = Ph, R^6 = H

76a, R^5, R^6 = (⁻CH$_2$⁻)$_4$; 48%
76b, R^5, R^6 = H; 31%
76c, R^5 = Ph, R^6 = H; 47%
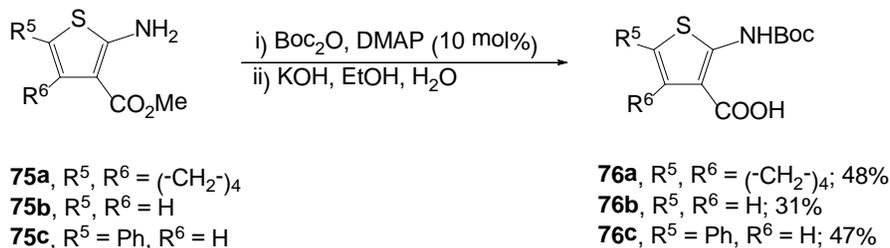
Scheme 26. Synthesis of **76a-c**

The synthetic method was designed to allow rapid access to TDZs in just three steps from the variable precursor building blocks. Initially, we tried the Ugi reaction of **76a**, *tert*-butyl isocyanide, formaldehyde, with glycine methyl ester hydrochloride in the presence of triethylamine under room temperature for 48 hours. After simple extraction workup, the intermediate Ugi product was subject to deprotection with TFA and subsequent cyclization using a catalytic amount of TBD. The product **77a** was isolated by chromatography in 46% yield over three steps. The 'three-step, one-separation' procedure was applied for the synthesis of **77a-e** with variable isocyanides and oxo components (**Table 13**). Interestingly, compounds **77f** and **77g**

were obtained in two steps after the treatment with TFA. It's possible that the intramolecular cyclization is favorable even without the treatment of TBD (29).

Table 13. UDC approach for the synthesis of 1,4-thienodiazepine-2,5-diones from glycine methyl ester
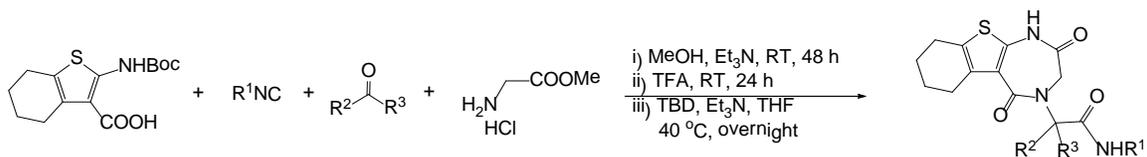
entry	R^1	oxo component	product	yield
77a	*t*-Bu	formaldehyde		46%[a]
77b	cyclopropyl methyl	formaldehyde		17%[a]
77c	phenyl ethyl	formaldehyde		13%[a]
77d	*t*-Bu	isobutylaldehyde		15%[a]
77e	*t*-Bu	cyclohexanone		10%[a]

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77f	t-Bu	o-chloro-benzaldehyde		37%[b]
77g	t-Bu	benzylaldehyde		21%[b]

[a] Method A, isolated yields (over three steps); [b] Method B, isolated yields (over two steps).

Next, we investigated a series of amino acid methyl esters for the synthesis of new TDZ scaffolds. We tried the Ugi reaction of **76a**, *tert*-butyl isocyanide, formaldehyde, with valine methyl ester hydrochloride in the presence of triethylamine under room temperature for 24 hours (**Scheme 27**). The Ugi product **78** was isolated by chromatography in 69% yield. The deprotection of **78** and the following cyclization afford **79a** in 59% yield over two steps (41% over three steps). Running the sequence without any isolation yielded compound **79a** 23%. Although the overall yield is lower, the 'three-step, one-separation' procedure avoids the additional separation step.

Scheme 27. Synthesis of **79a**

Hence, **76a** was applied to UDC approach for the synthesis of TDZs with variable amino acid derivatives (**Table 14**). Valine, phenylalanine, leucine, 4-hydroxy phenylglycine, trptophan methyl esters as well as several isocyanides are tolerant to this procedure. Compounds **79a-h** were isolated by chromatography in 14-37% yield over three steps. Since TBD serves as the catalyst, racemization of chiral center at amino acid nucleus is unclear.[285]

Table 14. 1,4-Thienodiazepine-2,5-diones from the variation of amino acids
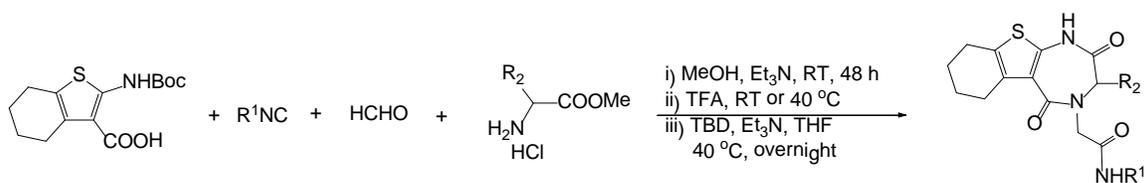
entry	R^1	amino acid	product	yield
79a	*t*-Bu	valine		23%^{b}

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79b	cyclopropyl methyl	valine		21%[b]
79c	benzyl	valine		24%[b]
79d	*t*-Bu	phenylalanine		21%[a]
79e	cyclopropyl methyl	phenylalanine		37%[a]
79f	*t*-Bu	leucine		14%[a]
79g	*t*-Bu	4-hydroxy phenylglycine		23%[a]
79h	*t*-Bu	trptophan		16%[a]
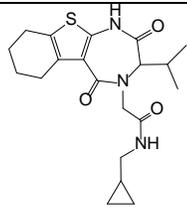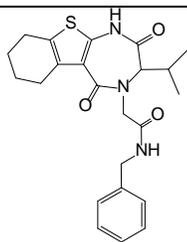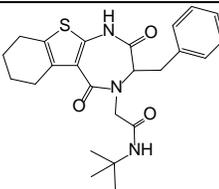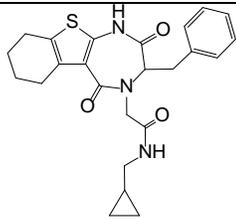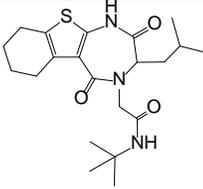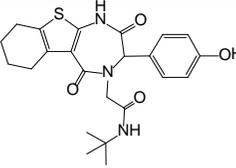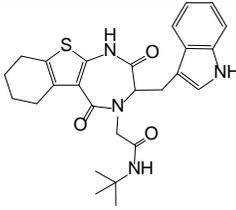
[a] Method A, isolated yields (over three steps); [b] Method C, isolated yields (over three steps).

We also investigated other aminothiophene backbones **76b** and **76c** for the synthesis of TDZs (**Table 15**). The corresponding Ugi product of **76b** was isolated by chromatography in 46% yield. Compound **80a** was obtained in 16% yield by the further transformation of the Ugi product. A 10% solution of TFA in dichloromethane was used for the deprotection step under a mild condition. Similarly, compound **80b** was isolated by chromatography in 11% yield over three steps.

Table 15. 1,4-Thienodiazepine-2,5-diones from the variation of aminothiophenes
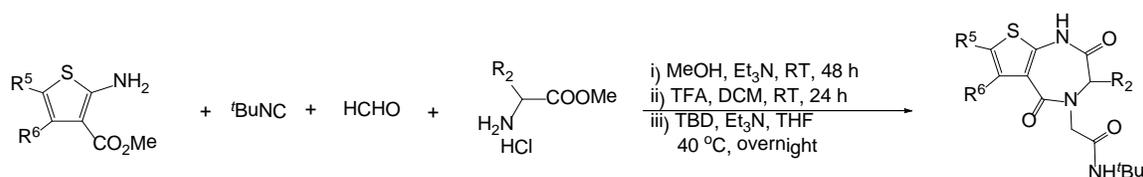
entry	R^5	R^6	amino acid	product	yield[a]
80a	H	H	valine		7%
80b	Ph	H	glycine		11%

[a] isolated yields, over three steps.

Conformation analysis of TDZ scaffold

NMR spectra of some compounds substituted with amino acid side chains show clearly the presence of two rotamers, which are not chromatographically separable. For instance, compounds **79d, 79e, 79h** show two rotamers at a ratio of 1:1 in CDCl$_3$. The population of

rotamers was found related to the deuterium solvent used. For example, the ratio of **79d** shows roughly 2:3 in CD_3OD. This observation suggests that seven-membered diazepine nucleus of thienodiazepinedione is quite rigid, similar to the scaffold of benzodiazepinediones.[286, 287] The energy barrier for the interconversion of benzodiazepinedione conformers (pseudo-axial and pseudo-equatorial conformers) was calculated up to 14.8 kcal/mol.[272] We also speculate that 1,4-thienodiazepine ring inversion is slow enough, therefore conformers can be detected by NMR under room temperature.

Recently, we used MCR methods to develop α-helix mimetics, which could become very important lead structures to (ant-)agonize PPIs.[288] In our ongoing interest for the application of peptidomimetic structures generated from MCRs, the design and synthesis of peptide β-turn mimetic scaffolds and libraries are also desirable.[289] A β-turn is most often defined as any tetrapeptide unit occurring in a nonhelical region that causes a reversal of the direction of the peptide chain (**Figure 45A**).[290] Since 1,4-benzodiazepines were found to act as β-turn mimetics,[268-270] we speculate that our TDZ scaffold could also have β-turn mimetic moiety. Thus, the tripeptide fragment of TDZ scaffold was compared with known protein β-turns (**Figure 45**). The core scaffold was investigated as a model superimposed onto a type II β-turn backbone (PDB code: 1H2C, turn region Ile142-Pro146).
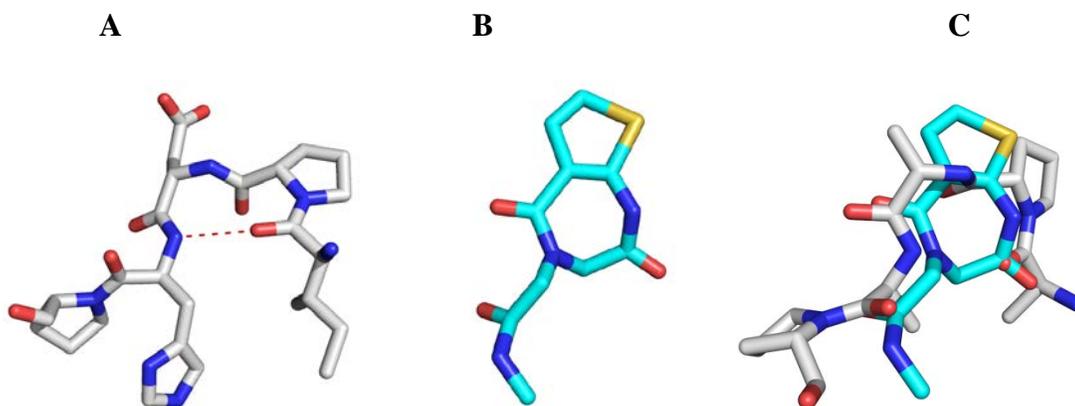
Figure 45. 1,4-thienodiazepine-2,5-dione as β-turn mimetic. (A) Structure of a typical β-turn. The PDB code for the protein is 1H2C (chain A, turn region Ile142-Pro146). (B) The core scaffold was chosen as a model of the investigated β-turn mimetic. (C) Minimized conformation of core scaffold as a β-turn mimetic superimposed onto a type II β-turn. For clarity, only the backbone and α atoms are shown.

Cheminformatics study of TDZ scaffold

Due to in silico and computational advances, chemoinformatics would help to identify promising scaffolds of greater importance in lead discovery.[291] We generated a virtual compound library (N = 50,000) from a random sample of starting materials to evaluate the chemical space of the TDZs. These compounds generated from the virtual library were introduced into Instant JChem (Instant JChem 2.5.1, 2009, www.chemaxon.com) for calculating their physical properties. The distributions of this random virtual library (N = 50,000) and commercial available benzodiazepines (N = 2,498) were presented in **Figure 46**. This new scaffold covers unexplored chemical space of benzodiazepine family. Due to the diversity of chemical space, this scaffold is potentially useful for virtual screening and lead discovery.
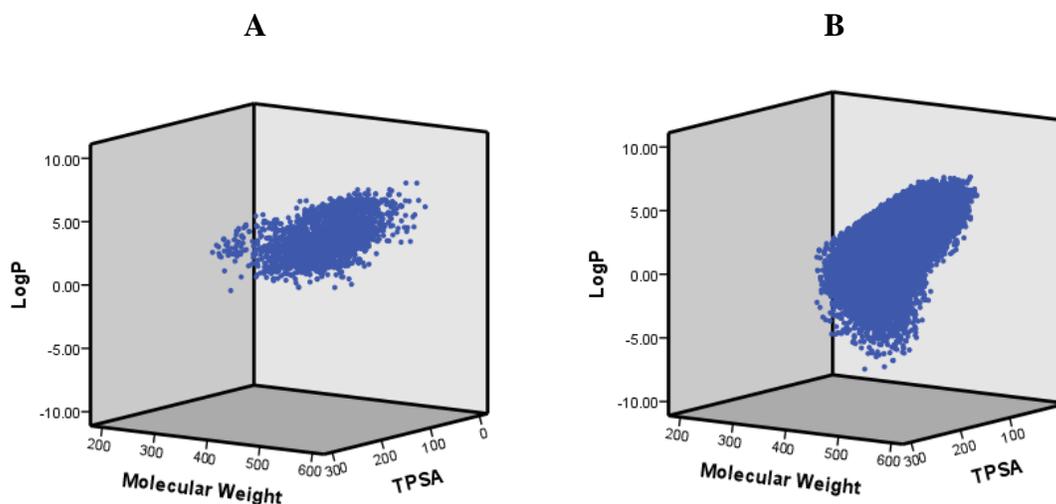
Figure 46. Comparison of chemical space distribution: (A) benzodiazepine library of a substructure search from eMolecules (N = 2,498); (B) a random virtual library of 1,4-thienodiazepine-2,5-diones (N = 50,000).

In order to evaluate the potential of combinatorial library design, the physicochemical properties of the virtual compound library (N = 50,000) were calculated.[292] The data was analyzed statistically by frequency distributions in PASW Statistics 18. Due to the diversity of reactant components, the range of molecular weight is between 267.3 Da. and 580.7 Da. The mean of LogP is 2.46 with standard deviation of 1.84, which indicates acceptable permeability of most compounds. TPSA of the majority of compounds is between 122.3 Å^2 and 176.9 Å^2. The mean number of rotatable bonds (NRB), hydrogen bond acceptors (HBA), and hydrogen bond donors (HBD) are shown in Table 4. In terms of drug likeness, 79.7% of 50,000 compounds obey Lipinski's rule. And 93.7% of them are predicted to be bioavailable (mass $\leq$ 500, LogP $\leq$ 5, HBD $\leq$ 5, HBA $\leq$ 10, PSA $\leq$ 200, NRB $\leq$ 10, fused aromatic rings $\leq$ 5). For comparison, the physicochemical properties of synthesized compounds (N = 17) and a random virtual library (N = 50,000) were summarized in **Table 16**. The structure-property relationship indicates the rationale of diversity-oriented library design with drug-like properties. Moreover, 3D structures

of a random virtual library were generated by the software Omega. The compound library of this new thienodiazepine scaffold (N = 5,000) could be used for docking program and other virtual analysis software to discover possible hits of suitable receptors.

Table 16. Physicochemical properties of synthesized compounds (N = 17) and a random virtual library (N = 50,000)[a]

Mean	MW (Da.)	LogP	TPSA ($\mathring{A}^2$)	NRB	HBA	HBD
synthesized compounds	407.1 ± 40.7	3.73 ± 0.94	108.9 ± 6.0	4 ± 1	4 ± 0	2 ± 0
virtual library	453.7 ± 44.1	2.46 ± 1.84	149.6 ± 27.3	7 ± 2	6 ± 1	3 ± 1

[a] All values listed are mean value plus/minus standard deviation.

Scaffold Hopping

Isofunctional molecules based on different chemical scaffolds are key to the early drug development process. Leads based on different scaffolds can be found by a process called scaffold hopping.[293] This process can rely on known or intuitive bioisosteres or on advanced chemoinformatic strategies. Early development of several leads based on different scaffolds has the advantage of reducing the very high attrition rate in preclinical and early clinical development. Additionally, scaffold hopping has great implications for the maintenance of intellectual property. For example, for the benzodiazepine scaffold 4672 structures are registered in SciFinder, whereas only 381 thienodiazepines are known (**Figure 47A**). We described here two thienodiazepine scaffolds with different 2D and 3D distribution of hydrogen bond donors and acceptors. They are clearly related to their benzodiazepine scaffolds amenable by the UDC

method (**Figure 47B,C**). The chemical space behind the four related scaffolds is however very much different, since the thiophene building block allows for many more simple variations based on the versatile Gewald MCR.

A

B

C

Armstrong et al.[131, 244]
Hulme et al.[247]

Tempest et al.[294]

Marcaccini et al.[295]

Zhu et al.[296]
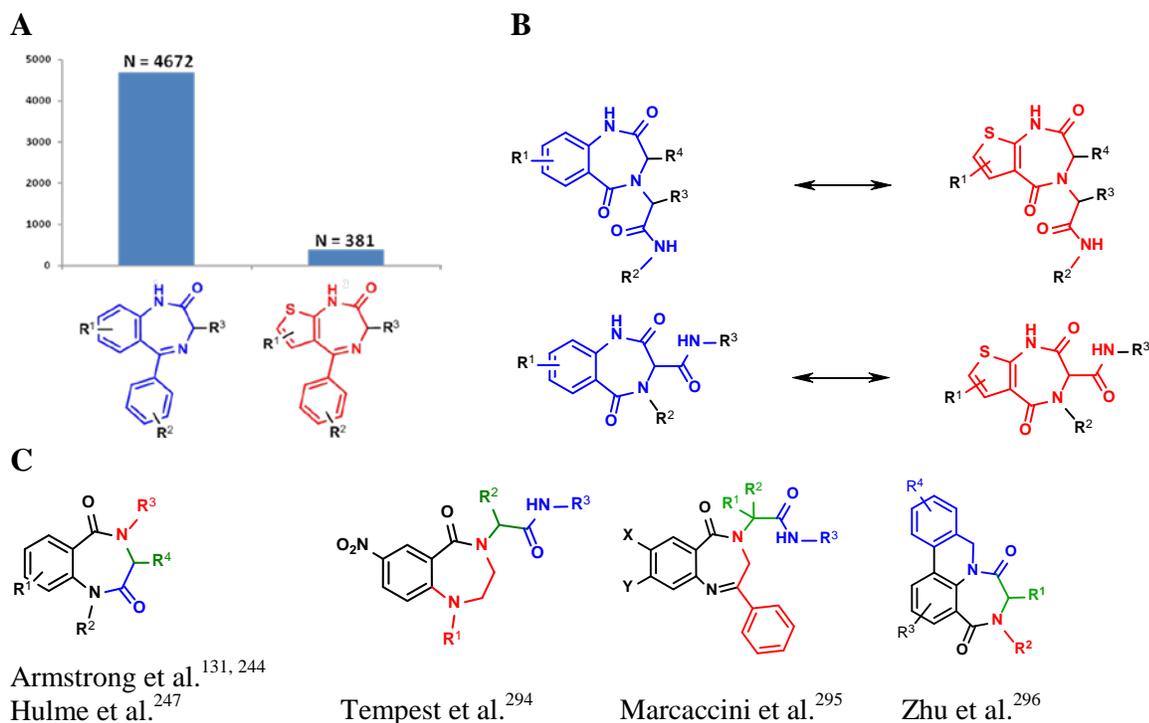
Figure 47. Scaffold hopping. (A) Known structures; (B) MCR acessible isosteric azepine scaffolds; (C) Other MCR accessible benzodiazepines. The scaffold space of benzodiazepines accessible by MCR is very rich. Six different scaffolds can be synthesized using different IMCR strategies. Of the bioisosteric thienodiazepines currently only two scaffolds are generally amenable by MCR.

In summary, we have synthesized a series of TDZs using the union of Gewald reaction and UDC strategy. This approach possesses novel hybrid peptidomimetic 1,4-diazepines with well-defined diversity, which can be achieved from readily available starting materials. UDC strategy allows convenient preparation of TDZs without using the traditional peptide coupling methods. Similar to the compound libraries of benzodiazepines, the TDZ scaffold could also be suitable for high-dimensional combinatorial synthesis to meet the screening purpose. The

conformation analysis and chemical space of this novel scaffold was studied. Based on the commonly accepted descriptors, it's potentially useful to obtain lead-like compounds based on this scaffold.

Materials and Methods

***N*-(*tert*-Butyl)-2-(2,5-dioxo-1,2,3,5,6,7,8,9-octahydro-4*H*-[1]benzothieno[2,3-e][1,4] diazepin-4-yl)acetamide (77a, Method A):** The mixture of **76a** (59.4 mg, 0.2 mmol), glycine methyl ester hydrochloride (0.2 mmol, 25.0 mg), triethylamine (0.2 mmol, 27.9 µL), aqueous formaldehyde (0.2 mmol, 14.9 µL), *tert*-butyl isocyanide (0.2 mmol, 22.6 µL) in 0.5 mL of methanol was stirring under RT for 2 days. The reaction was quenched by water, and extracted by DCM. The organic layer was washed by saturated potassium carbonate (aq), and dried over anhydrous sodium sulfate. After the evaporation of the solvent, the residue was treated by 0.5 mL of TFA, stirring under RT for 24 h. The reaction was added 10 mL of DCM, then neutralized by saturated potassium carbonate (aq). The mixture was extracted by DCM, the organic layer was combined and dried over anhydrous sodium sulfate. After the evaporation of the solvent, the residue was treated by triethylamine (50 µL) and TBD (10 mg) in 0.5 mL of THF, stirring overnight under 40 ^{o}C. **77a** was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellowish solid (32 mg, yield: 46% over three steps). HPLC/MS: t_R = 9.42 min; m/z = 350.1 [M+H]$^{+}$. HRMS: 349.145979 (found); $C_{17}H_{23}N_3O_3S$, 349.14601 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 1.33 (9H, s), 1.77 (2H, m), 1.83 (2H, m), 2.62 (2H, m), 2.76 (2H, m), 4.08 (2H, s), 4.12 (2H, s), 6.21 (1H, s). ^{13}C NMR (150 MHz, CDCl$_3$): 22.3, 23.0, 24.5, 25.8, 28.7, 51.5, 52.5, 52.7, 121.7, 129.2, 135.0, 141.4, 164.4, 167.4, 169.0.

***N*-(Cyclopropylmethyl)-2-(2,5-dioxo-1,2,3,5,6,7,8,9-octahydro-4*H*-[1]benzothieno[2,3-e][1,4]diazepin-4-yl)acetamide (77b, Method A):** The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellowish solid (12 mg, yield: 17% over three steps). HPLC/MS: t_R = 9.12 min; m/z = 348.1 [M+H]$^+$. HRMS: 347.131963 (found); $C_{17}H_{21}N_3O_3S$, 347.13036 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 0.19 (2H, m), 0.48-0.50 (2H, m), 0.94 (1H, m), 1.77-1.84 (4H, m), 2.64 (2H, m), 2.77 (2H, m), 3.11 (2H, m), 4.10 (2H, s), 4.21 (2H, s), 6.49 (1H, s). ^{13}C NMR (150 MHz, CDCl$_3$): 3.4, 10.5, 22.3, 23.0, 24.5, 25.8, 44.3, 52.0, 52.5, 121.7, 129.2, 135.1, 141.3, 164.5, 168.0, 168.8.

***N*-(2-Phenylethyl)-2-(2,5-dioxo-1,2,3,5,6,7,8,9-octahydro-4*H*-[1]benzothieno[2,3-e][1,4]diazepin-4-yl)acetamide (77c, Method A):** The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellowish solid (10 mg, yield: 13% over three steps). HPLC/MS: t_R = 9.81 min; m/z = 398.1 [M+H]$^+$. HRMS: 397.145798 (found); $C_{21}H_{23}N_3O_3S$, 397.14601 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 1.77-1.83 (4H, m), 2.63 (2H, m), 2.73 (2H, m), 2.80 (2H, m), 3.51 (2H, m), 3.99 (1H, s), 4.15 (2H, s), 6.48 (1H, m), 7.17-7.21 (3H, m), 7.25-7.28 (2H, m). ^{13}C NMR (150 MHz, CDCl$_3$): 22.3, 23.0, 24.5, 25.8, 35.5, 40.6, 52.1, 52.4, 121.5, 126.5, 128.6, 128.8, 129.1, 135.0, 138.7, 141.6, 164.5, 168.2, 168.7.

***N*-(*tert*-Butyl)-2-(2,5-dioxo-1,2,3,5,6,7,8,9-octahydro-4*H*-[1]benzothieno[2,3-e][1,4]diazepin-4-yl)-3-methylbutanamide (77d, Method A):** The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellowish solid (12 mg, yield: 15% over three steps). HPLC/MS: t_R = 10.69 min; m/z = 392.2 [M+H]$^+$. HRMS: 391.192703 (found); $C_{20}H_{29}N_3O_3S$, 391.19296 (calcd.). ^{1}H NMR (600 MHz, d^6-DMSO): 0.72 (3H, d, J = 6.0 Hz), 0.89 (3H, d, J = 6.6 Hz), 1.23 (9H, s), 1.61-1.78 (5H, m), 2.10 (1H, m), 2.58 (2H, m), 3.16 (1H, m), 3.86 (1H, d, J = 14.4 Hz), 4.63 (1H, d, J = 10.8 Hz), 7.72 (1H, s). ^{13}C NMR (150 MHz, d^6-

DMSO): 19.2, 19.6, 22.5, 23.1, 24.4, 26.1, 28.0, 28.8, 47.2, 50.7, 50.8, 121.0, 127.2, 134.5, 159.9, 164.2, 169.9.

N-(*tert*-Butyl)-1-(2,5-dioxo-1,2,3,5,6,7,8,9-octahydro-4*H*-[1]benzothieno[2,3-e][1,4] diazepin-4-yl)cyclohexanecarboxamide (77e, Method A): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellowish solid (8 mg, yield: 10% over three steps). HPLC/MS: t_R = 11.00 min; m/z = 418.3 [M+H]$^+$. HRMS: 440.1981 (found); $C_{22}H_{31}N_3NaO_3S$, 440.19783 (calcd.). ^{1}H NMR (600 MHz, CD$_3$OD): 1.34 (9H, s), 1.45-1.51 (3H, m), 1.61-1.65 (2H, m), 1.81-1.92 (6H, m), 2.03 (2H, m), 2.23 (1H, m), 2.45 (1H, m), 2.67 (2H, m), 2.97 (1H, m), 4.11 (2H, s), 6.55 (1H, s). ^{13}C NMR (150 MHz, CD$_3$OD): 22.2, 22.8, 23.9, 25.1, 25.7, 27.48, 27.50, 46.8, 50.7, 50.8, 66.69, 66.71, 122.6, 128.4, 134.4, 142.1, 165.4, 170.7, 174.3.

N-(*tert*-Butyl)-2-(4-chlorophenyl)-2-(2,5-dioxo-1,2,3,5,6,7,8,9-octahydro-4*H*-[1] benzothieno[2,3-e][1,4]diazepin-4-yl)acetamide (77f, Method B): The mixture of **6a** (59.4 mg, 0.2 mmol), glycine methyl ester hydrochloride (0.2 mmol, 25.0 mg), triethylamine (0.2 mmol, 27.9 µL), *o*-chloro-benzylaldehyde (0.2 mmol, 28.0 mg), *tert*-butyl isocyanide (0.2 mmol, 22.6 µL) in 0.5 mL of methanol was stirring under RT for 2 days. The reaction was quenched by water, and extracted by DCM. The organic layer was washed by saturated potassium carbonate (aq), and dried over anhydrous sodium sulfate. After the evaporation of the solvent, the residue was treated by 0.5 mL of TFA, stirring under RT for 24 h. The reaction was added 10 mL of DCM, then neutralized by saturated potassium carbonate (aq). The mixture was extracted by DCM, the organic layer was combined and dried over anhydrous sodium sulfate. After the evaporation of the solvent, **77f** was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellowish solid (34 mg, yield: 37% over two steps). HPLC/MS: t_R = 11.08 min;

m/z = 460.1 [M+H]$^+$. HRMS: 459.137649 (found); C$_{23}$H$_{26}$ClN$_3$O$_3$S, 459.13834 (calcd.). ^{1}H NMR (600 MHz, CD$_3$OD): 1.31-1.34 (2H, m), 1.38 (9H, s), 1.74-1.90 (4H, m), 2.67 (2H, m), 3.23 (1H, m), 3.87 (1H, m), 6.22 (1H, s), 7.33 (2H, d, *J* = 7.8 Hz), 7.43 (2H, d, *J* = 7.8 Hz), 7.94 (1H, s). ^{13}C NMR (150 MHz, CD$_3$OD): 7.8, 22.2, 22.8, 23.9, 25.5, 27.4, 46.5, 51.2, 120.7, 128.2, 128.8, 130.8, 134.1, 134.2, 134.4, 142.8, 165.1, 169.3, 169.6.

N-(*tert*-Butyl)-2-(2,5-dioxo-1,2,3,5,6,7,8,9-octahydro-4*H*-[1]benzothieno[2,3-e][1,4] diazepin-4-yl)-3-phenylpropanamide (77g, Method B): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellowish solid (18 mg, yield: 21% over two steps). HPLC/MS: t_R = 11.03 min; *m/z* = 440.3 [M+H]$^+$. HRMS: 439.193597 (found); C$_{24}$H$_{29}$N$_3$O$_3$S, 439.19296 (calcd.). ^{1}H NMR (600 MHz, *d*^{6}-DMSO): 0.86 (1H, s), 1.22 (9H, s), 1.61-1.69 (4H, m), 2.55 (2H, s), 2.88 (1H, m), 3.14 (1H, m), 3.96 (1H, m), 5.36 (1H, s), 7.22 (1H, m), 7.26 (3H, m), 7.29 (1H, m), 7.44 (1H, m), 7.51 (1H, s). ^{13}C NMR (150 MHz, *d*^{6}-DMSO): 22.4, 23.1, 24.3, 26.0, 28.2, 28.8, 35.8, 48.0, 50.9, 57.6, 120.7, 126.6, 127.1, 128.4, 129.4, 134.6, 137.9, 164.0, 169.7.

Methyl 2-(2-(*tert*-butoxycarbonylamino)-N-(2-(*tert*-butylamino)-2-oxoethyl)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxamido)-3-methylbutanoate (78): The mixture of **76a** (74.3 mg, 0.25 mmol), valine methyl ester hydrochloride (0.25 mmol, 41.8 mg), triethylamine (0.25 mmol, 34.8 µL), aqueous formaldehyde (0.25 mmol, 18.6 µL), *tert*-butyl isocyanide (0.25 mmol, 28.3 µL) in 0.5 mL of methanol was stirring under RT for 1 day. The reaction was quenched by water, and extracted by DCM. The organic layer was washed by saturated potassium carbonate (aq), and dried over anhydrous sodium sulfate. After the evaporation of the solvent, the product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 3:1) as yellowish solid (90 mg, yield: 69%). HRMS: 523.272509 (found); C$_{26}$H$_{41}$N$_3$O$_6$S,

523.27161 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$): 0.82 (1H, d, J = 6.0 Hz), 0.85 (1H, d, J = 6.6 Hz), 1.36 (9H, s), 1.50 (9H, s), 1.74-1.82 (4H, m), 2.05 (1H, m), 2.24 (1H, m), 2.62-2.65 (3H, m), 3.68 (3H, s), 3.86 (1H, d, J = 15.0 Hz), 4.02 (1H, d, J = 10.2 Hz), 4.62 (1H, d, J = 15.6 Hz), 5.53 (1H, s), 10.03 (1H, s). ^{13}C NMR (150 MHz, CDCl$_3$): 18.9, 19.4, 22.6, 23.4, 24.0, 28.2, 28.3, 28.6, 29.6, 45.0, 51.8, 52.1, 66.3, 80.3, 126.6, 129.7, 137.5, 153.0, 167.6, 168.8, 170.6.

N-($tert$-Butyl)-2-(3-isopropyl-2,5-dioxo-1,2,3,5,6,7,8,9-octahydro-4H-[1]benzothieno[2,3-e][1,4]diazepin-4-yl)acetamide (79a, Method C): The mixture of **76a** (74.3 mg, 0.25 mmol), valine methyl ester hydrochloride (0.25 mmol, 41.8 mg), triethylamine (0.25 mmol, 34.8 µL), aqueous formaldehyde (0.25 mmol, 18.6 µL), $tert$-butyl isocyanide (0.25 mmol, 28.3 µL) in 0.5 mL of methanol was stirring under RT for 2 days. The reaction was quenched by water, and extracted by DCM. The organic layer was washed by saturated potassium carbonate (aq), and dried over anhydrous sodium sulfate. After the evaporation of the solvent, the residue was treated by 0.5 mL of TFA, stirring under 40 °C overnight. The reaction was added 10 mL of DCM, then neutralized by saturated potassium carbonate (aq). The mixture was extracted by DCM, the organic layer was combined and dried over anhydrous sodium sulfate. After the evaporation of the solvent, the residue was treated by triethylamine (50 µL) and TBD (10 mg) in 0.5 mL of THF, stirring overnight under 40 °C. **79a** was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellowish solid (22 mg, yield: 23% over three steps). HPLC/MS: t_R = 10.44 min, m/z = 392.3 [M+H]$^+$. HRMS: 391.192778 (found); C$_{20}$H$_{29}$N$_3$O$_3$S, 391.19296 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$, major rotamer): 0.92 (1H, d, J = 6.6 Hz), 0.96 (1H, d, J = 6.6 Hz), 1.32 (9H, s), 1.69 (1H, m), 1.77 (1H, m), 1.89-1.96 (3H, m), 2.43 (1H, m), 2.60-2.68 (2H, m), 3.07 (1H, m), 3.70 (1H, d, J = Hz), 4.07-4.16 (2H, ABd, J = Hz), 6.66 (1H, s). ^{13}C NMR (150

MHz, CDCl$_3$, major rotamer): 19.4, 20.0, 23.0, 24.5, 25.8, 26.7, 28.6, 51.3, 57.3, 73.8, 122.0, 128.8, 134.7, 140.1, 163.6, 167.6, 170.0.

N-(Cyclopropylmethyl)-2-(3-isopropyl-2,5-dioxo-1,2,3,5,6,7,8,9-octahydro-4*H*-[1] benzothieno[2,3-e][1,4]diazepin-4-yl)acetamide (79b, Method C): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 1:1) as yellowish solid (16 mg, yield: 21% over three steps). HPLC/MS: t_R = 10.10 min; m/z = 490.3 [M+H]$^+$. HRMS: 389.176195 (found); C$_{20}$H$_{27}$N$_3$O$_3$S, 389.17731 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$, major rotamer): 0.16-0.19 (2H, m), 0.46- 0.48 (2H, m), 0.88 (3H, d, J = 6.6 Hz), 0.92-0.94 (1H, m), 0.95 (3H, d, J = 6.6 Hz), 1.66-1.76 (2H, m), 1.88-1.96 (3H, m), 2.43-2.46 (1H, m), 2.59-2.66 (2H, m), 3.02-3.08 (2H, m), 3.11-3.14 (1H, m), 3.71 (1H, d, J = 11.4 Hz), 4.17 (1H, ABd, J = 15.0 Hz), 4.25 (1H, ABd, J = 15.0 Hz), 6.91 (1H, s). ^{13}C NMR (150 MHz, CDCl$_3$, major rotamer): 3.4, 3.5, 10.6, 19.1, 20.0, 22.3, 23.0, 24.5, 25.9, 26.7, 44.3, 56.1, 73.8, 121.8, 128.6, 134.7, 140.7, 163.7, 168.4, 169.9.

N-Benzyl-2-(3-isopropyl-2,5-dioxo-1,2,3,5,6,7,8,9-octahydro-4*H*-[1]benzothieno[2,3-e][1,4]diazepin-4-yl)acetamide (79c, Method C): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 1:1) as yellowish solid (20 mg, yield: 24% over three steps). HPLC/MS: t_R = 10.50 min; m/z = 426.3 [M+H]$^+$. HRMS: 425.177499 (found); C$_{23}$H$_{27}$N$_3$O$_3$S, 425.17731 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$, major rotamer): 0.79 (3H, d, J = 6.6 Hz), 0.90 (3H, d, J = 6.0 Hz), 1.65-1.72 (2H, m), 1.83-1.93 (3H, m), 2.35-2.38 (1H, m), 2.57-2.61 (1H, m), 2.62-2.63 (1H, m), 2.98-3.00 (1H, m), 3.68 (1H, d, J = 11.4 Hz), 4.11 (1H, d, J = 15.0 Hz), 4.33-4.45 (3H, m), 7.22-7.31 (6H, m). ^{13}C NMR (150 MHz, CDCl$_3$, major rotamer): 19.2, 19.9, 22.3, 22.9, 24.5, 25.8, 26.7, 43.5, 56.2, 74.0, 121.7, 127.3, 127.8, 128.6, 128.8, 134.6, 137.9, 140.8, 163.8, 168.5, 169.8.

***N*-(*tert*-Butyl)-2-(3-benzyl-2,5-dioxo-1,2,3,5,6,7,8,9-octahydro-4*H*-[1]benzothieno[2,3-e][1,4]diazepin-4-yl)acetamide (79d, Method A):** The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 1:1) as yellowish solid (18 mg, yield: 21% over three steps). HPLC/MS: t_R = 10.89 min; m/z = 440.2 [M+H]$^+$. HRMS: 439.194543 (found); $C_{24}H_{29}N_3O_3S$, 439.19296 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$, 1:1 mixture of rotamers): 1.30 (9H, s), 1.34 (9H, s), 1.71-1.81 (4H, m), 1.91-1.95 (4H, m), 2.52-2.66 (6H, m), 2.97 (2H, m), 3.10 (2H, m), 3.20 (1H, m), 3.62 (2H, m), 3.88 (1H, m), 4.09 (1H, m), 4.19 (1H, m), 4.35 (1H, m), 4.49 (1H, m), 5.99 (1H, s), 6.47 (1H, s), 7.05-7.06 (2H, m), 7.21-7.28 (8H, m). ^{13}C NMR (150 MHz, CDCl$_3$, 1:1 mixture of rotamers): 22.3, 22.4, 22.99, 23.03, 24.58, 24.60, 25.5, 26.2, 28.59, 28.63, 32.6, 34.6, 48.3, 51.3, 51.4, 55.4, 57.7, 68.1, 122.3, 122.5, 126.9, 127.3, 128.5, 128.6, 128.9, 129.0, 129.1, 129.45, 129.50, 134.7, 135.0, 135.6, 136.6, 140.2, 141.4, 163.2, 165.5, 167.4, 168.3, 168.4, 169.6.

***N*-(Cyclopropylmethyl)-2-(3-benzyl-2,5-dioxo-1,2,3,5,6,7,8,9-octahydro-4*H*-[1]benzothieno[2,3-e][1,4]diazepin-4-yl)acetamide (79e, Method A):** The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 1:1) as yellowish solid (32 mg, yield: 37% over three steps). HPLC/MS: t_R = 10.53 min; m/z = 438.2 [M+H]$^+$. HRMS: 460.1687 (found); $C_{24}H_{27}N_3NaO_3S$, 460.16708 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$, 1:1 mixture of rotamers): 0.12 (2H, m), 0.16-0.18 (2H, m), 0.40-0.42 (2H, m), 0.45-0.46 (2H, m), 0.84-0.95 (2H, m), 1.75-1.77 (4H, m), 1.90-1.93 (4H, m), 2.55-2.63 (6H, m), 2.92-2.96 (2H, m), 2.98-3.02 (2H, m), 3.06-3.08 (4H, m), 3.25 (1H, m), 3.56 (1H, m), 3.67-3.75 (2H, m), 4.02 (1H, m), 4.13 (1H, m), 4.22 (1H, m), 4.38 (1H, m), 4.51 (1H, m), 6.44 (1H, m), 6.85 (1H, m), 7.04-7.05 (2H, m), 7.19-7.28 (8H, m). ^{13}C NMR (150 MHz, CDCl$_3$, 1:1 mixture of rotamers): 3.3, 3.4, 10.5, 10.6, 22.3, 22.4, 23.00, 23.04, 24.6, 25.5, 26.2, 32.6, 34.3, 41.0, 44.3, 47.2, 52.1, 54.8, 55.7, 57.8, 68.3, 122.2,

122.3, 126.9, 127.3, 128.57, 128.60, 128.8, 128.9, 129.1, 129.3, 129.4, 129.5, 134.6, 134.8, 135.7, 136.6, 140.7, 141.8, 163.4, 165.7, 168.1, 168.5, 168.8, 169.6.

N-(*tert*-Butyl)-2-(3-isobutyl-2,5-dioxo-1,2,3,5,6,7,8,9-octahydro-4*H*-[1]benzothieno[2,3-e][1,4]diazepin-4-yl)acetamide (79f, Method A): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 1:1) as yellowish solid (11 mg, yield: 14% over three steps). HPLC/MS: t_R = 10.87 min; *m/z* = 406.2 [M+H]$^+$. HRMS: 405.209380 (found); $C_{21}H_{31}N_3O_3S$, 405.20861 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 0.88-0.91 (6H, m), 0.93-0.98 (6H, m), 1.34 (9H, s), 1.37 (5H, s), 1.47-1.50 (2H, m), 1.57-1.65 (3H, m), 1.71-1.75 (3H, m), 1.80-1.82 (2H, m), 1.90-1.93 (4H, m), 2.09 (1H, m), 2.45-2.54 (2H, m), 2.63-2.71 (3H, m), 2.92 (1H, m), 3.07-3.11 (2H, m), 3.22 (1H, m), 3.32 (1H, m), 3.79 (1H, d, *J* = 15.6 Hz), 3.94 (1H, d, *J* = 15.0 Hz), 4.12 (1H, d, *J* = 15.6 Hz), 4.18 (1H, m), 4.22 (1H, m), 4.31 (1H, d, *J* = 15.0 Hz), 6.35 (1H, s), 6.46 (1H, s). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 22.0, 22.26, 22.28, 22.32, 22.4, 22.7, 22.8, 22.96, 23.02, 24.55, 24.59, 25.0, 25.1, 25.5, 25.7, 26.0, 28.6, 28.7, 35.0, 37.9, 42.7, 48.3, 50.5, 51.2, 51.3, 51.7, 52.0, 54.8, 56.1, 60.1, 65.1, 122.4, 122.7, 128.7, 129.5, 134.8, 135.0, 139.7, 141.0, 163.5, 165.9, 167.6, 168.2, 168.8, 170.4.

N-(*tert*-Butyl)-2-[3-(4-hydroxyphenyl)-2,5-dioxo-1,2,3,5,6,7,8,9-octahydro-4*H*-[1] benzothieno[2,3-e][1,4]diazepin-4-yl]acetamide (79g, Method A): Sodium bicarbonate was used for the workup instead of saturated potassium carbonate (aq). The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 1:1) as yellowish solid (20 mg, yield: 23% over three steps). HPLC/MS: t_R = 9.53 min; *m/z* = 442.2 [M+H]$^+$. HRMS: 441.173542 (found); $C_{23}H_{27}N_3O_4S$, 441.17223 (calcd.). ^{1}H NMR (600 MHz, CD$_3$OD): 1.36 (9H, s), 1.50 (1H, m), 1.67-1.77 (3H, m), 2.22 (1H, m), 2.46 (2H, m), 2.70 (1H, m), 2.85 (1H, m), 3.22 (1H, m), 4.13 (1H, m), 4.64 (1H, m), 5.22 (1H, m), 6.62 (2H, m), 6.97 (2H, m), 7.62 (1H, m). ^{13}C

NMR (150 MHz, CD$_3$OD): 22.0, 22.7, 23.7, 25.2, 27.6, 34.1, 43.3, 51.0, 53.3, 63.5, 68.5, 114.8, 123.2, 124.2, 125.2, 128.2, 133.4, 140.3, 156.8, 164.7, 167.7, 170.6.

N-(*tert*-Butyl)-2-[3-(1*H*-indol-3-ylmethyl)-2,5-dioxo-1,2,3,5,6,7,8,9-octahydro-4*H*-[1] benzothieno [2,3-e][1,4]diazepin-4-yl]acetamide (79h, Method A): The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 1:1) as yellowish solid (15 mg, yield: 16% over three steps). HPLC/MS: t_R = 10.68 min; m/z = 479.3 [M+H]$^+$. HRMS: 478.203784 (found); C$_{26}$H$_{30}$N$_4$O$_3$S, 478.20386 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$, 1:1 mixture of rotamers): 1.32 (9H, s), 1.38 (9H, s), 1.68-1.96 (8H, m), 2.53-2.65 (6H, m), 3.11-3.20 (4H, m), 3.36 (1H, m), 3.68 (1H, m), 3.86 (1H, m), 3.99-4.03 (2H, m), 4.18 (1H, m), 4.48-4.50 (2H, m), 6.01 (1H, s), 6.38 (1H, s), 6.89 (1H, s), 7.05 (1H, s), 7.09-7.13 (3H, m), 7.18 (1H, m), 7.25 (1H, d, *J* = 7.8 Hz), 7.34 (1H, d, *J* = 7.8 Hz), 7.53 (1H, d, *J* = 7.8 Hz), 7.58 (1H, d, *J* = 7.8 Hz), 8.27 (1H, s), 8.52 (1H, s). ^{13}C NMR (150 MHz, CDCl$_3$, 1:1 mixture of rotamers): 22.2, 22.3, 22.4, 23.0, 24.3, 24.5, 24.6, 25.6, 26.3, 28.6, 28.7, 48.0, 51.36, 51.44, 55.6, 56.9, 67.1, 109.3, 110.2, 111.3, 111.4, 118.17, 118.23, 119.6, 122.00, 122.04, 122.1, 122.5, 123.4, 123.8, 126.8, 127.0, 128.8, 129.5, 134.6, 135.1, 135.9, 136.1, 140.0, 141.1, 163.3, 165.7, 167.6, 168.2, 168.7, 170.0.

N-(*tert*-Butyl)-2-(3-isopropyl-2,5-dioxo-1,2,3,5-tetrahydro-4*H*-thieno[2,3-e][1,4]diazepin-4-yl)acetamide (80a): The mixture of **76b** (48.6 mg, 0.2 mmol), valine methyl ester hydrochloride (0.2 mmol, 33.5 mg), triethylamine (0.2 mmol, 27.9 µL), aqueous formaldehyde (0.2 mmol, 14.9 µL), *tert*-butyl isocyanide (0.2 mmol, 22.6 µL) in 0.5 mL of methanol was stirring under RT for 2 days. The Ugi product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 5:1), then treated by 0.5 mL of DCM (10% TFA). The reaction mixture was stirring under RT for 24 hours. After the evaporation of the solvent, the residue was treated by triethylamine (100 µL) and TBD (10 mg) in 0.5 mL of THF, stirring overnight under 40 ^{o}C. The

product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 1:1) as yellowish solid (5 mg, yield: 7% over three steps). HPLC/MS: t_R = 8.99 min; m/z = 338.3 [M+H]$^+$. HRMS: 337.145879 (found); $C_{16}H_{23}N_3O_3S$, 337.14601 (calcd.). ^{1}H NMR (600 MHz, CDCl$_3$, major rotamer): 0.94 (1H, d, J = 6.6 Hz), 0.98 (1H, d, J = 6.6 Hz), 1.34 (9H, s), 1.95 (1H, m), 3.75 (1H, d, J = 9.6 Hz), 4.03 (1H, d, J = 15.0 Hz), 4.27 (1H, d, J = 15.0 Hz), 6.44 (1H, m), 6.87 (1H, d, J = 6.0 Hz), 8.60 (1H, br.s). ^{13}C NMR (150 MHz, CDCl$_3$, major rotamer): 19.4, 19.9, 27.2, 28.6, 57.2, 73.7, 117.0, 123.8, 128.1, 142.3, 163.2, 167.0, 169.0.

N-(*tert*-Butyl)-2-(2,5-dioxo-7-phenyl-1,2,3,5-tetrahydro-4*H*-thieno[2,3-e][1,4]diazepin-4-yl)acetamide (80b): The mixture of **76c** (63.8 mg, 0.2 mmol), glycine methyl ester hydrochloride (0.2 mmol, 25.0 mg), triethylamine (0.2 mmol, 27.9 µL), aqueous formaldehyde (0.2 mmol, 14.9 µL), *tert*-butyl isocyanide (0.2 mmol, 22.6 µL) in 0.5 mL of methanol was stirring under RT for 2 days. The reaction was quenched by water, and extracted by DCM. The organic layer was washed by saturated potassium carbonate (aq), and dried over anhydrous sodium sulfate. After the evaporation of the solvent, the residue was treated by 1.0 mL of DCM (10% TFA), stirring under RT for 24 h. After the evaporation of the solvent, the residue was treated by triethylamine (200 µL) and TBD (10 mg) in 0.5 mL of THF, stirring overnight under 40 ^{o}C. The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellowish solid (8 mg, yield: 11% over three steps). HPLC/MS: t_R = 9.66 min; m/z = 372.1 [M+H]$^+$. HRMS: 371.128759 (found); $C_{19}H_{21}N_3O_3S$, 371.13036 (calcd.). ^{1}H NMR (600 MHz, CD$_3$OD): 1.37 (9H, s), 4.09 (2H, s), 4.21 (2H, s), 7.31-7.33 (1H, m), 7.40-7.42 (2H, m), 7.47 (1H, s), 7.58-7.59 (2H, m). ^{13}C NMR (150 MHz, CD$_3$OD): 27.5, 50.9, 51.1, 52.9, 122.3, 123.3, 124.9, 127.7, 128.8, 133.0, 135.3, 165.0, 167.7, 168.7.

3.4   DESIGN AND SYNTHESIS OF 1,4-BENZODIAZEPINES LIBRARIES

Encouraged by the examples shown in **Chapter 3.3**, novel IMCR strategies were developed to generate pilot-scale libraries based on the previlidged structure 1,4-benzodiazepine. In **Chapter 3.4.1**, diversre 1,4-benzodiazepine scaffolds were designed and synthesized, which provide the templates to introduce generate drug-like compounds, preferentially targeting PPIs. In **Chapter 3.4.2**, key "anchor" residues, which are aboundant in the PPI interface, were incorporated to a 1,4-benzodiazepine scaffold. These anchor-biased compounds would recognize the PPI interface involved by the deeply buried "anchor" residues, and potentially disrupt the PPI with biological relevance.

3.4.1   Diversity-oriented Synthesis of Novel Benzodiazepine Scaffolds

The synthesis and evaluation of new scaffolds based on privileged structures play an important role in the drug discovery process. Privileged structures are often preferentially chosen by medicinal chemists, since there is the believe that the "spirit" of an approved and successful compound based on such a scaffold can be transferred into a new compound for a different indication.[297] Benzodiazepines are a family of drugs that are used to relieve insomnia and anxiety, as well as to treat muscles spasm and prevent seizures.[298] They are one of the most widely prescribed medications for the central nervous system. Notably, diazepam is one of the most frequently prescribed medications in the world during the past 40 years. Over 40 medications highlight benzodiazepine as a classic privileged structure with a broad range of therapeutic treatment.[299, 300] After the discovery of benzodiazepine family, many synthetic derivatives with a wide pharmacological spectrum have been extensively developed.[301] The

development of new synthetic approaches to benzodiazepines has attracted considerable attention in the discovery of biologically active compounds.

The chemical syntheses of benzodiazepines have been extensively investigated since 1960s.[302] In the early 1990s, Ellman and et al. developed a general method for the solid-phase synthesis of 1,4-benzodiazepine libraries.[303] As shown in **Scheme 28**, 2-aminobenzophenone derivatives **81** were first attached to the polystyrene solid support, and transformed to 1,4-benzodiazepine derivatives **85** by constitute linear syntheses over five steps. The benzodiazepine products **86** were cleaved from the support in very high overall yields. The library of 1,4-benzodiazepine derivatives was evaluated by a screening assay against cholecystokinin A receptor.[304]
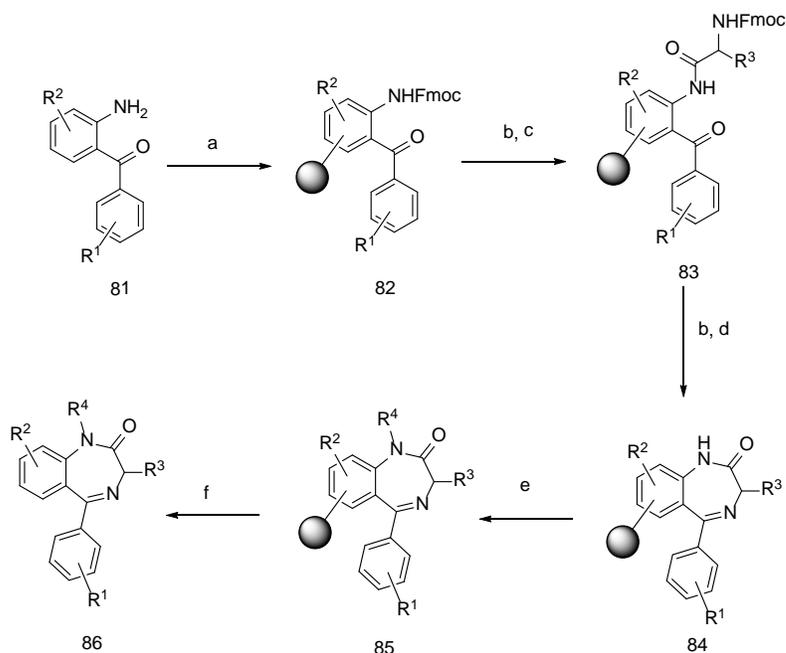
Scheme 28. Ellman's 1,4-benzodiazepine synthesis

In contrast to constitute linear syntheses, MCR chemistry allows for the synthesis of arrays of compounds in a highly efficient and diverse manner.[305, 306] MCRs allow the resource

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and cost effective, fast, and convergent synthesis of diverse compound libraries, and highly improve the efficiency to explore the chemical space with limited synthetic effort.[307] IMCRs, such as the Ugi and Passerini reactions, provide a powerful tool for producing arrays drug-like compounds with high atom economy.[102] Post-condesnsation reactions of MCR products have been extensively investigated for the construction of heterocycles.[308] During the past decades, IMCR chemistry has been applied to synthesize diverse libraries based on benzodiazepine scaffolds as drug-like compounds, such as modulators of germ cell nuclear factor (GCNF) to regulate stem cell differentiation.[309] In general, benzodiazepine heterocyclic cores **92** can be achieved by the Ugi reaction of bifunctional starting materials in conjunction with post-condensation cyclization (**Figure 48**).
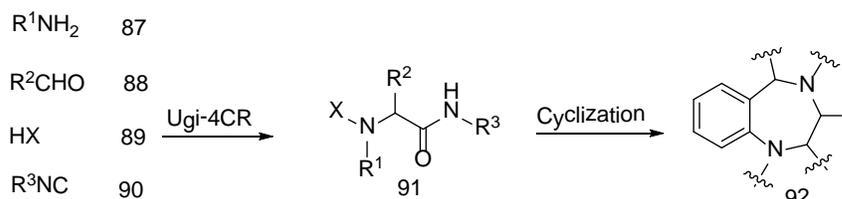
Figure 48. General strategy for the synthesis of benzodiazepines via Ugi-MCRs

Up to date, IMCRs have been successfully applied for the synthesis of benzodiazepines in an efficient and diverse manner.[310] These innovative approaches have become increasingly popular as tools for the rapid generation of drug-like benzodiazepine libraries. Taking advantages of the concise and powerful synthetic methodologies, this strategy will fulfill the drug discovery efforts on the privilege scaffold. In the near future, rational design of novel MCRs will become much more important to discover uncovered chemical space of

benzodiazepines. The collection of diverse benzodiazepine libraries representing enormous chemical space will provide a useful toolbox in structure-based drug design and drug discovery.

N-Boc-α-amino-aldehydes have been shown as bifunctional starting materials for MCRs.[223, 311] Hulme and coworkers utilized the Ugi reaction with N-Boc-α-amino-aldehydes for a solution phase synthesis of an array of biologically relevant imidazolines and azepine-tetrazoles.[312, 313] Transformations were carried out in excellent yield utilizing Ugi/de-Boc/cyclization (UDC) strategy in a remarkable 'three-step-one-pot' procedure. Herein, we developed novel applications of Boc-glycinal with the Ugi MCR for the synthesis of 1,4-benzodiazepines.

Anthranilic acid derivatives have shown reseanable reactivity for Ugi-4CRs.[314-316] Methyl anthranilate was employed as the building block for the synthesis of 1,4-benzodiazepine scaffold **93** (**Scheme 29**). In the first step, methyl anthranilate serves as an amine component for Ugi four-componment reaction with Boc-glycinal, isocyanide, and acid. The Boc protection group of Boc-glycinal is cleaved in the second step. The free amine is condensed with ester group of methyl anthranilate to form 1,4-diazepine ring in the third step. The UDC strategy allows to access 1,4-benzodiazepines with different substitutions in Scheme 29.
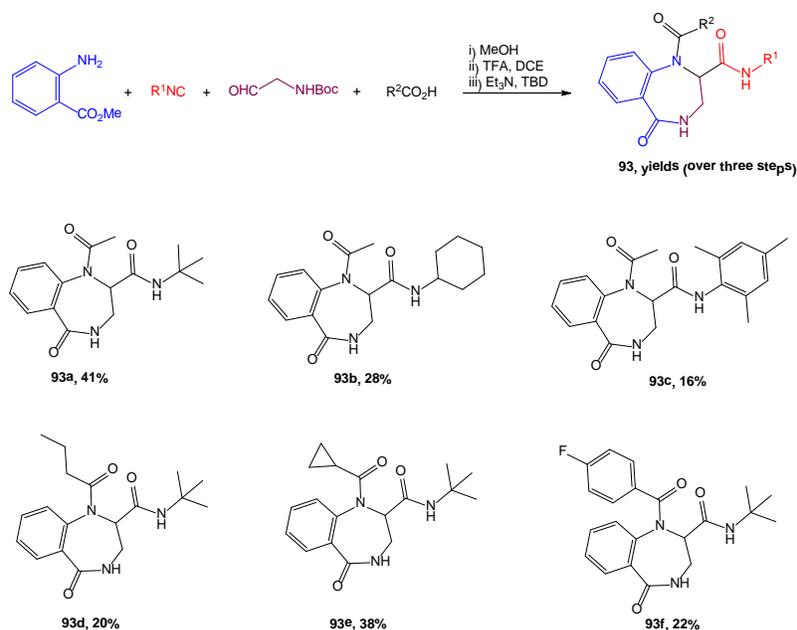
Scheme 29. Synthesis of scaffold 1

Aminophenylketones have shown good reactivity for MCRs as an amine component.[317-319] Aminophenylketones were commonly employed as the building block for the synthesis of 1,4-benzodiazepine scaffold.[320] UDC strategy was applied to aminophenylketones for the rapid access 1,4-benzodiazepines **94**. In the first step, aminophenylketones serve as an amine component for Ugi four-component reaction with Boc-glycinal, isocyanide, and acid (**Scheme 30, condition A**). Microwave was utilized for the Ugi 4CR to reduce the reaction time (**Scheme 30, condition B**).[321] In the second step, the deprotected amino group is immediately cyclized with the ketone functionality to form 1,4-diazepine ring. A small focused library with three points of diversity was obtained using this versatile methodlogy (**Scheme 30**).
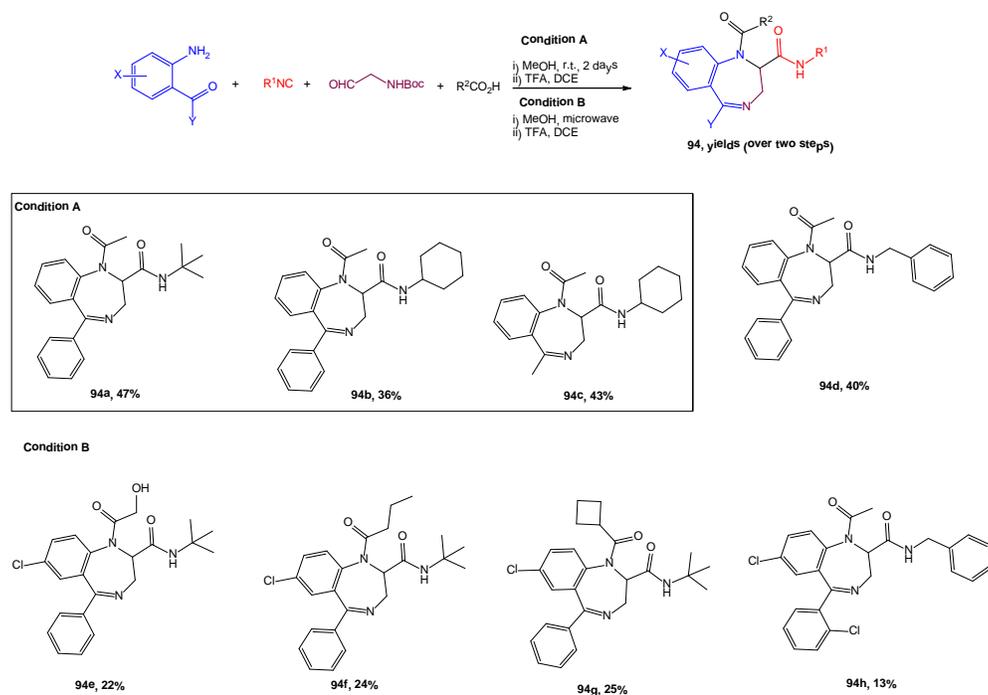
Scheme 30. Synthesis of scaffold 2

UDC strategy was applied for the synthesis of third 1,4-benzodiazepine scaffold **95**. In the first step, aminophenylketones serve as an amine component for Ugi four-component reaction with Boc-glycinal, isocyanide, and azide (**Scheme 31, condition A**). Microwave assisted Ugi reaction (**Scheme 31, condition B**) proceeded in a reaction time of only 30 min compared to the conventional methodology which required up to 48 h. In the second step, the deprotected amino group is immediately cyclized with the ketone functionality to form 1,4-diazepine ring. A group of 2-tetrazole substituted 1,4-benzodiazepines were synthesized (**Scheme 31**).
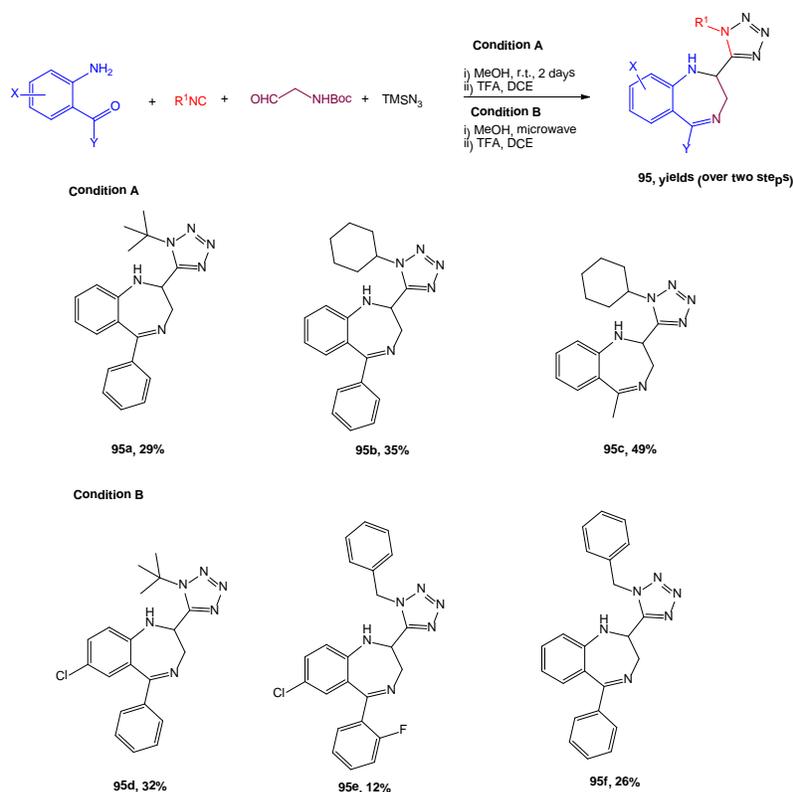
Scheme 31. Synthesis of scaffold 3

In summary, three diverse 1,4-benzodiazepine scaffolds were designed and synthesized, which allow to address unexplored drug-like chemical space. Methyl anthranilate and aminophenylketones were first used as the building block for the synthesis of 1,4-benzodiazepine scaffold via Ugi-4CRs. Boc-glycinal was employed as the bifunctional starting materials, thus "anchor" fragments can be introduced to 1,4-benzodiazepines using substituted α-amino-aldehydes.

Materials and Methods

Representative procedure for the synthesis of 1,4-benzodiazepines:

Preparation of **1-acetyl-*N*-tert-butyl-5-oxo-2,3,4,5-tetrahydro-1*H*-benzo[e][1,4]diazepine-2-carboxamide (93a, Method A)**: The mixture of methyl anthranilate (0.2 mmol, 26.0 μL), Boc-

glycinal (0.2 mmol, 31.8 mg), tert-butyl isocyanide (0.2 mmol, 22.6 µL), acetic acid (0.2 mmol, 11.5 µL) and 0.5 mL of methanol was stirring for 2 days under room temperature. The Ugi product was isolated by silica gel chromatography (hexanes/ethyl acetate, 1:1) as yellowish solid. The Ugi product was treated by 0.5 mL of DCM (10% TFA), and the reaction mixture was stirring under RT for 2 days. After the evaporation of the solvent, the residue was treated by triethylamine (100 µL) and TBD (10 mg) in 0.5 mL of THF, stirring overnight under 40 °C. The product was isolated by silica gel chromatography (ethyl acetate) as white solid (25 mg, yield: 41% over three steps). HPLC/MS: t_R = 8.66 min; m/z = 304.3 [M+H]$^+$. HRMS: $C_{16}H_{21}N_3O_3Na$, 326.1481 (calcd.), 326.1507 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.35 (s, 9H), 1.88 (s, 3H), 3.43-3.54 (m, 2H), 5.25-5.28 (m, 1H), 6.75 (s, 1H), 7.11 (m, 1H), 7.26 (m, 1H), 7.53-7.59 (m, 2H), 7.79 (m, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 23.0, 28.7, 40.5, 51.3, 62.4, 129.4, 129.7, 129.9, 132.6, 132.7, 136.4, 167.6, 170.9, 172.6.

Preparation of **1-acetyl-*N*-cyclohexyl-5-oxo-2,3,4,5-tetrahydro-1*H*-benzo[e][1,4]diazepine-2-carboxamide (93b, Method A):** The product was isolated by silica gel chromatography (ethyl acetate / hexanes, 5:1) as yellowish solid (21 mg, yield: 28% over three steps). SFC/MS: t_R = 2.29 min; m/z = 370.32 [M-H]$^-$. HRMS: $C_{21}H_{29}N_3O_3Na$, 394.2107 (calcd.), 394.2097 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.88-1.18 (m, 3H), 1.35 (s, 9H), 1.50- 1.75 (m, 6 H), 2.04-2.05 (m, 1H), 3.41-3.43 (m, 1H), 3.49 (m, 2H), 5.26-5.29 (m, 1H), 6.77(s, 1H), 6.84 (s, 1H), 7.06-7.08 (m, 1H), 7.56-7.58 (m, 2H), 7.80-7.82 (m, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 25.2, 25.4, 25.5, 28.7, 29.47, 29.48, 40.3, 42.0, 51.2, 61.8, 128.8, 129.7, 129.9, 132.5, 133.1, 135.9, 167.8, 170.8, 178.7.

Preparation of **1-acetyl-*N*-mesityl-5-oxo-2,3,4,5-tetrahydro-1*H*-benzo[e][1,4]diazepine-2-carboxamide (93c, Method A):** The product was isolated by silica gel chromatography (ethyl acetate / hexanes, 5:1) as white solid (10 mg, intermediate); white solid (12 mg, yield: 16% over

three steps). HRMS: $C_{21}H_{23}N_3O_3Na$, 388.1637(calcd.), 388.1712(found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.96 (s, 3H), 2.21 (s, 6H), 2.29 (s, 3H), 3.59-3.67 (m, 2H), 5.57-5.60 (m, 1H), 6.77 (m, 1H), 6.91 (s, 2H), 7.24-7.29 (m, 1H), 7.56-7.60 (m, 2H), 7.85 (m, 1H), 8.22 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 18.5, 20.9, 23.0, 40.6, 62.2, 129.0, 129.5, 129.8, 130.2, 130.6, 132.6, 132.7, 134.5, 136.2, 137.0, 166.8, 170.5, 173.0.

Preparation of *N-tert*-**butyl-1-butyryl-5-oxo-2,3,4,5-tetrahydro-1*H*-benzo[e][1,4]diazepine-2-carboxamide (93d, Method A):** The product was isolated by silica gel chromatography (ethyl acetate / hexanes, 5:1) as yellow oil (13 mg, yield: 20% over three steps). SFC/MS: t_R = 2.16 min; m/z = 330.25 [M-H]$^-$. HRMS: $C_{18}H_{25}N_3O_3Na$, 354.1794 (calcd.), 354.1793 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.81-0.84 (m, 3H), 1.36 (s, 9H), 1.54-1.62 (m, 2H), 1.92-1.97 (m, 1H), 2.10-2.14 (m, 1H), 3.42-3.46 (m, 1H), 3.49-3.52 (m, 1H), 5.27-5.30 (m, 1H), 6.79 (s, 1H), 6.94 (s, 1H), 7.08 (m, 1H), 7.54-7.58 (m, 2H), 7.80-7.82 (m, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 13.5, 18.7, 28.7, 36.4, 40.3, 51.3, 62.1, 129.4, 129.7, 129.9, 132.7, 133.0, 136.0, 167.7, 170.8, 175.3.

Preparation of *N-tert*-**butyl-1-(cyclopropanecarbonyl)-5-oxo-2,3,4,5-tetrahydro-1*H*-benzo[e][1,4]diazepine-2-carboxamide (93e, Method A):** The product was isolated by silica gel chromatography (ethyl acetate / hexanes, 5:1) as white solid (25 mg, yield: 38% over three steps). SFC/MS: t_R = 2.25 min; m/z = 328.24 [M-H]$^-$. HRMS: $C_{18}H_{23}N_3O_3Na$, 352.1637 (calcd.), 352.1625 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.69 (m, 1H), 0.78-0.82 (m, 2H), 0.92 (m, 1H), 1.18-1.21 (m, 2H), 1.34 (s, 9H), 3.45-3.48 (m, 1H), 3.55-3.58 (m, 1H), 5.21-5.24 (m, 1H), 6.92 (s, 1H), 7.14-7.16 (m, 1H), 7.22 (m, 1H), 7.29 (s, 1H), 7.52-7.54 (m, 1H), 7.56-7.58 (m, 1H), 7.81-7.83 (m, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 7.5, 8.0, 9.0, 10.4, 13.6, 14.1, 28.71, 28.74, 40.4, 44.9, 51.0, 51.2, 62.5, 129.4, 129.8, 129.9, 132.6, 133.0, 136.2, 167.8, 171.1, 172.0, 176.0, 177.9.

Preparation of ***N*-tert-butyl-1-(4-fluorobenzoyl)-5-oxo-2,3,4,5-tetrahydro-1*H*-benzo[e][1,4] diazepine-2-carboxamide (93f, Method A):** The product was isolated by silica gel chromatography (ethyl acetate / hexanes, 5:1) as yellowish solid (17 mg, yield: 30% over two steps). SFC/MS: t_R = 2.47 min; m/z = 382.23 [M-H]⁻. HRMS: $C_{21}H_{22}FN_3O_3Na$, 406.1543(calcd.), 406.1479 (found). ^{1}H NMR (600 MHz, CDCl₃): 1.41 (s, 9H), 3.54-3.58 (m, 1H), 3.74-3.77 (m, 1H), 5.27-5.30 (m, 1H), 6.61 (m, 1H), 6.65 (m, 1H), 6.87-6.88 (m, 2H), 7.13 (s, 1H), 7.21-7.26 (m, 2H), 7.29 (s, 1H), 7.38-7.41 (m, 1H), 7.80 (m, 1H). ^{13}C NMR (150 MHz, CDCl₃): 28.8, 40.7, 51.4, 63.2, 115.3, 115.4, 129.1, 129.7, 129.9, 130.4, 131.0, 131.1, 132.5, 132.8, 136.9, 167.3, 170.9, 171.4.

Preparation of **2-(1-*tert*-butyl-1*H*-tetrazol-5-yl)-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4] diazepine (95a, Method B)**: The mixture of 2-aminobenzophenone (0.2 mmol, 39.4 mg), Boc-glycinal (0.2 mmol, 31.8 mg), *tert*-butyl isocyanide (0.2 mmol, 22.6 µL), TMSN₃ (0.3 mmol, 39.5 µL) and 0.5 mL of methanol was stirring for 2 days under room temperature. The Ugi product was isolated by silica gel chromatography (hexanes/ethyl acetate, 5:1) as yellow solid (62 mg). The mixture of the Ugi product (26 mg), and 0.5 mL of DCE (10% TFA) was stirring under 40 ^{o}C overnight. The residue was treated by triethylamine (100 µL), and the product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 3:1) as yellowish solid (20 mg, yield: 29% over two steps). SFC/MS: t_R = 2.98min; m/z = 347.26 [M+H]⁺. HRMS: $C_{20}H_{22}N_6Na$, 369.1804 (calcd.), 369.1805 (found). ^{1}H NMR (600 MHz, CDCl₃): 1.83 (s, 9H), 4.04 (m, 1H), 4.24 (m, 1H), 4.33 (m, 1H), 5.61 (m, 1H), 6.92-6.97 (m, 2H), 7.12 (m, 1H), 7.34-7.48 (m, 4H), 7.61-7.63 (m, 2H). ^{13}C NMR (150 MHz, CDCl₃): 30.3, 55.3, 59.2, 61.7, 120.1, 121.1, 123.9, 128.2, 129.4, 130.1, 131.8, 131.9, 140.5, 145.8, 155.5, 174.1.

Preparation of **2-(1-cyclohexyl-1*H*-tetrazol-5-yl)-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4] diazepine (95b, Method B):** The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 3:1) as yellowish solid (26 mg, yield: 35% over two steps). HPLC/MS: t_R = 8.39 min; m/z = 372.6 [M+H]$^+$. HRMS: $C_{22}H_{25}N_6$, 373.2141 (calcd.), 373.2160 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.62-0.64 (m, 1H), 1.16 (m, 2H), 1.30-1.35 (m, 1H), 1.47-1.52 (m, 4H), 1.79-1.82 (m, 3H), 1.87-1.96 (m, 6H), 2.24-2.26 (m, 1H), 4.00-4.03 (m, 1H), 4.08-4.11 (m, 1H), 4.49-4.52 (m, 2H), 4.56-4.61 (m, 1H), 5.61-5.63 (m, 1H), 7.00 (m, 1H), 7.07 (m, 1H), 7.13 (m, 1H), 7.36-7.41 (m, 3H), 7.44-7.47 (m, 2H). ^{13}C NMR (150 MHz, CDCl$_3$): 24.6, 24.7, 24.8, 24.9, 25.2, 32.6, 33.1, 33.2, 54.3, 58.6, 58.8, 61.6, 121.0, 124.9, 128.2, 129.4, 130.4, 131.5, 131.8, 139.9, 140.6, 146.0, 154.7, 174.2.

Preparation of **2-(1-cyclohexyl-1*H*-tetrazol-5-yl)-5-methyl-2,3-dihydro-1*H*-benzo[e][1,4] diazepine (95c, Method B):** The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 3:1) as yellow solid (31 mg, yield: 49% over two steps). HPLC/MS: t_R = 7.33 min; m/z = 311.3 [M+H]$^+$. HRMS: $C_{17}H_{23}N_6$, 311.1984 (calcd.), 311.1988 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.24-1.34 (m, 2H), 1.68 (m, 2H), 1.85-1.99 (m, 6H), 2.40 (s, 3H), 3.69-3.72 (m, 1H), 3.86 (m, 1H), 4.27 (s, 1H), 4.37-4.41 (m, 1H), 5.36 (m, 1H), 6.86 (d, 1H, *J* = 7.8 Hz), 6.92-6.95 (m, 1H), 7.22-7.26 (m, 1H), 7.32 (d, 1H, *J* = 7.8 Hz). ^{13}C NMR (150 MHz, CDCl$_3$): 24.8, 25.3, 25.4, 27.0, 33.1, 33.2, 53.9, 58.5, 59.6, 104.4, 120.8, 121.0, 125.4, 129.0, 131.7, 144.2, 154.6, 173.6.

Preparation of **2-(1-tert-butyl-1*H*-tetrazol-5-yl)-7-chloro-5-phenyl-2,3-dihydro-1*H*-benzo[e] [1,4]diazepine (95d, Method C):** The mixture of 2-amino-5-chlorobenzophenone (0.2 mmol, 46.2 mg), Boc-glycinal (0.2 mmol, 31.8 mg), tert-butyl isocyanide (0.2 mmol, 22.6 μL), TMSN3 (0.3 mmol, 39.5 μL) and 0.5 mL of methanol was stirring under microwave irradiation (100 ^{o}C,

30 min). The Ugi product was isolated by silica gel chromatography (hexanes/ethyl acetate, 5:1) as yellow solid. The mixture of the Ugi product, and 0.5 mL of DCE (10% TFA) was stirring under 40 $^\circ$C overnight. The residue was treated by triethylamine (100 µL), and the product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 3:1) as yellow solid (24 mg, yield: 32% over two steps). SFC/MS: t_R = 2.99 min; m/z = 381.22 [M+H]$^+$. HRMS: $C_{20}H_{22}ClN_6$, 381.1594 (calcd.), 381.1629 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.67 (s, 9H), 3.73 (t, 1H, J = 10.8 Hz), 4.47 (s, 1H), 4.50-4.52 (dd, 1H, J = 11.4, 3.6 Hz), 5.34-5.36 (dd, 1H, J = 10.8, 3.6 Hz), 6.81 (d, 1H, J = 8.4 Hz), 6.70 (m, 1H), 7.16-7.19 (m, 2H), 7.32-7.40 (m, 3H), 7.53 (d, 1H, J = 7.2 Hz). ^{13}C NMR (150 MHz, CDCl$_3$): 29.4, 54.4, 60.6, 64.2, 122.2, 124.9, 125.8, 128.2, 129.2, 130.2, 130.8, 131.2, 139.9, 145.0, 166.2, 172.0.

Preparation of **2-(1-benzyl-1*H*-tetrazol-5-yl)-7-chloro-5-(2-fluorophenyl)-2,3-dihydro-1*H*-benzo[e][1,4]diazepine (95e, Method C):** The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 3:1) as yellow solid (10 mg, yield: 12% over two steps). SFC/MS: t_R = 3.48 min; m/z = 433.20 [M+H]$^+$. HRMS: $C_{23}H_{19}ClFN_6$, 433.1344 (calcd.), 433.1388 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 4.11-4.21 (m, 2H), 5.52 (m, 1H), 5.57 (ABd, 1H, J = 15.6 Hz), 5.72 (ABd, 1H, J = 15.6 Hz), 6.79 (m, 1H), 7.02 (m, 1H), 7.12 (m, 1H), 7.23-7.26 (m, 3H), 7.32-7.35 (m, 3H), 7.44-7.52 (m, 3H). ^{13}C NMR (150 MHz, CDCl$_3$): 51.6, 54.4, 60.2, 122.0, 122.8, 123.0, 123.2, 124.5, 126.0, 127.5, 128.3, 129.0, 129.2, 130.0, 131.1, 132.0, 133.7, 143.2, 155.1.

Preparation of **2-(1-benzyl-1*H*-tetrazol-5-yl)-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4] diazepine (95f, Method C)**: The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 3:1) as yellow solid (20 mg, yield: 26% over two steps). SFC/MS: t_R = 3.28 min; m/z = 379.26 [M-H]$^-$. HRMS: $C_{23}H_{21}N_6$, 381.1828 (calcd.), 381.1866 (found). ^{1}H NMR

(600 MHz, CDCl$_3$): 4.02-4.10 (m, 3H), 5.50 (ABd, 1H, J = 15.0 Hz), 5.56-5.58 (m, 1H), 5.70 (ABd, 1H, J = 15.0 Hz), 6.88 (m, 1H), 7.00-7.02 (m, 1H), 7.13-7.17 (m, 3H), 7.32-7.38 (m, 4H), 7.40-7.48 (m, 4H), 7.60 (m, 2H). ^{13}C NMR (150 MHz, CDCl$_3$): 51.7, 53.9, 60.7, 121.0, 121.2, 125.1, 127.6, 128.3, 128.8, 129.1, 129.3, 129.5, 130.4, 131.6, 131.9, 133.8, 140.0, 145.4, 155.5, 174.4.

Preparation of **1-acetyl-*N*-*tert*-butyl-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepine-2-carboxamide (94a, Method D)**: The mixture of 2-aminobenzophenone (0.2 mmol, 39.4 mg), Boc-glycinal (0.2 mmol, 31.8 mg), tert-butyl isocyanide (0.2 mmol, 22.6 µL), acetic acid (0.2 mmol, 11.5 µL) and 0.5 mL of methanol was stirring for 2 days under room temperature. The product was isolated by silica gel chromatography (hexanes/ethyl acetate, 3:1) as yellow solid (66 mg). The isolated Ugi product and 0.5 mL of DCE (10% TFA) was stirring under 40 ^{o}C overnight. The residue was treated by triethylamine (100 µL), and the product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 1:1) as white solid (34 mg, yield: 47% over two steps). SFC/MS: t_R = 2.18 min; *m/z* = 362.26 [M-H]$^-$. HRMS: C$_{22}$H$_{25}$N$_3$O$_2$Na, 386.1844 (calcd.), 386.1855 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.38 (s, 9H), 1.91 (s, 9H), 3.51 (1H, t, J = 12.6 Hz), 4.12 (dd, 1H, J = 4.2 Hz, 13.2 Hz), 5.43 (dd, 1H, J = 4.2 Hz, 12.0 Hz), 6.71 (s, 1H), 7.24-7.26 (m, 2H), 7.40-7.43 (m, 2H), 7.47-7.50 (m, 2H), 7.54-7.56 (m, 1H), 7.64-7.65 (m, 2H). ^{13}C NMR (150 MHz, CDCl$_3$): 23.1, 28.7, 50.7, 51.2, 66.1, 128.5, 128.6, 128.7, 129.4, 129.7, 130.7, 131.4, 133.4, 138.2, 139.1, 168.0, 170.2, 171.5.

Preparation of **1-acetyl-*N*-cyclohexyl-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepine-2-carboxamide (94b, Method D)**: The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 1:1) as white solid (28 mg, yield: 36% over two steps). SFC/MS: t_R = 2.43 min; *m/z* = 388.31 [M-H]$^-$. HRMS: C$_{24}$H$_{28}$N$_3$O$_2$, 390.2182 (calcd.), 390.2228 (found).

¹H NMR (600 MHz, CDCl₃): 1.21-1.23 (m, 4H), 1.35-1.38 (m, 2H), 1.58-1.60 (m, 1H), 1.69-1.71 (m, 2H), 1.90 (s, 3H), 3.51 (t, 1H, *J* = 12.6 Hz), 3.74-3.77 (m, 1H), 4.13-4.16 (dd, 1H, *J* = 12.0, 4.2 Hz), 5.47-5.50 (dd, 1H, *J* = 13.2, 4.2 Hz), 6.75 (m, 1H), 7.24 (m, 2H), 7.40-7.42 (m, 2H), 7.46-7.49 (m, 2H), 7.52-7.55 (m, 1H), 7.63-7.65 (m, 2H). ¹³C NMR (150 MHz, CDCl₃): 23.1, 24.7, 25.5, 32.7, 33.0, 48.0, 50.8, 65.7, 128.5, 128.6, 128.7, 129.6, 130.8, 131.3, 133.4, 138.2, 139.1, 167.8, 170.3, 171.5.

Preparation of **1-acetyl-*N*-cyclohexyl-5-methyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepine-2-carboxamide (94c, Method D)**: The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 1:1) as yellowish solid (28 mg, yield: 43% over two steps). SFC/MS: t_R = 2.18 min; *m/z* = 328.25 [M+H]⁺. HRMS: $C_{19}H_{26}N_3O_2$, 328.2025 (calcd.), 328.2061 (found). ¹H NMR (600 MHz, CDCl₃): 1.20-1.21 (m, 4H), 1.34-1.37 (m, 2H), 1.58 (m, 1H), 1.69-1.71 (m, 2H), 1.83 (s, 3H), 1.87-1.89 (m, 2H), 2.34 (s, 3H), 3.31 (t, 1H, *J* = 13.2 Hz), 3.72 (m, 1H), 3.86-3.89 (dd, 1H, *J* = 12.6, 4.8 Hz), 5.38-5.41 (dd, 1H, *J* = 13.2, 4.8 Hz), 6.71 (m, 1H), 7.14 (m, 1H), 7.38-7.40 (m, 1H), 7.44-7.48 (m, 2H). ¹³C NMR (150 MHz, CDCl₃): 22.9, 24.7, 25.5, 26.0, 32.7, 33.0, 48.0, 50.1, 65.4, 127.1, 129.0, 129.5, 131.0, 134.9, 137.2, 167.8, 170.5, 171.7.

Preparation of **1-acetyl-*N*-benzyl-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepine-2-carboxamide (94d, Method E):** The mixture of 2-aminobenzophenone (0.2 mmol, 39.4 mg), Boc-glycinal (0.2 mmol, 31.8 mg), benzyl isocyanide (0.2 mmol, 24.4 µL), acetic acid (0.2 mmol, 11.5 µL) and 0.5 mL of methanol was stirring under microwave irradiation (100 ^oC, 30 min). The product was isolated by silica gel chromatography (hexanes/ethyl acetate, 3:1) as yellow solid (55 mg). The isolated Ugi product and 0.5 mL of DCE (10% TFA) was stirring under 40 ^oC overnight. The residue was treated by triethylamine (100 µL), and the product was

isolated by silica gel chromatography (petroleum ether/ethyl acetate, 1:1) as yellow solid (32 mg, yield: 40% over two steps). SFC/MS: t_R = 2.66 min; m/z = 398.26 [M+H]$^+$. HRMS: $C_{25}H_{24}N_3O_2$, 398.1869 (calcd.), 398.1908 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.87 (s, 3H), 3.55 (t, 1H, J = 12.6 Hz), 4.20-4.23 (dd, 1H, J = 12.0, 4.8 Hz), 4.38-4.41 (m, 1H), 4.51-4.54 (m, 1H), 5.56-5.59 (dd, 1H, J = 13.2, 4.8 Hz), 7.15 (m, 1H), 7.26 (m, 1H), 7.30-7.43 (m, 4H), 7.48-7.52 (m, 3H), 7.64-7.65 (m, 2H). ^{13}C NMR (150 MHz, CDCl$_3$): 23.0, 43.3, 50.8, 65.6, 127.4, 127.6, 128.5, 128.65, 128.68, 128.73, 129.6, 129.7, 130.8, 131.4, 133.3, 138.2, 138.3, 139.0, 168.8, 170.5, 171.7.

Preparation of **N-*tert*-butyl-7-chloro-1-(2-hydroxyacetyl)-5-phenyl-2,3-dihydro-1*H*-benzo[e] [1,4]diazepine-2-carboxamide (94e, Method E)**: The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 1:1) as yellow solid (18 mg, yield: 22% over two steps). SFC/MS: t_R = 2.38 min; m/z = 414.20 [M+H]$^+$. HRMS: $C_{22}H_{25}ClN_3O_3$, 414.1584 (calcd.), 414.1609 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.38 (s, 9H), 3.14 (br.s, 1H), 3.50 (t, 1H, J = 12.6 Hz), 3.60 (ABd, 1H, J = 15.6 Hz), 4.13-4.16 (dd, 1H, J = 12.0, 4.2 Hz), 4.18 (ABd, 1H, J = 16.8 Hz), 5.35-5.38 (dd, 1H, J = 13.2, 4.2 Hz), 6.27 (s, 1H), 7.27 (m, 2H), 7.43-7.46 (m, 2H), 7.51-7.55 (m, 2H), 7.62 (m, 2H). ^{13}C NMR (150 MHz, CDCl$_3$): 28.9, 50.7, 51.6, 61.0, 67.0, 128.5, 128.8, 129.8, 130.5, 131.3, 131.7, 134.6, 135.0, 135.7, 137.2, 167.0, 168.8, 172.8.

Preparation of **N-*tert*-butyl-1-butyryl-7-chloro-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4] diazepine-2-carboxamide (94f, Method E)**: The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 1:1) as yellow solid (20 mg, yield: 24% over two steps). SFC/MS: t_R = 2.09 min; m/z = 426.24 [M+H]$^+$. HRMS: $C_{24}H_{29}ClN_3O_2$, 426.1948 (calcd.), 426.1988 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.79 (m, 3H), 1.38 (s, 9H), 1.52-1.61 (m, 2H), 1.93-1.96 (m, 1H), 2.20-2.25 (m, 1H), 3.50 (t, 1H, J = 12.6 Hz), 4.11-4.14 (dd, 1H, J = 12.6, 4.8

Hz), 5.40-5.43 (dd, 1H, J = 13.2, 4.8 Hz), 6.63 (s, 1H), 7.17 (d, 1H, J = 8.4 Hz), 7.25 (s, 1H), 7.44-7.46 (m, 2H), 7.50-7.54 (m, 2H), 7.64 (m, 2H). ^{13}C NMR (150 MHz, CDCl$_3$): 13.6, 18.7, 28.7, 36.5, 50.7, 51.3, 66.0, 128.5, 128.6, 129.4, 130.8, 131.0, 131.4, 134.8, 135.0, 137.3, 137.5, 167.8, 168.9, 173.9.

Preparation of ***N*-*tert*-butyl-7-chloro-1-(cyclobutanecarbonyl)-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepine-2-carboxamide (94g, Method E)**: The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 1:1) as yellow solid (22 mg, yield: 25% over two steps). SFC/MS: t_R = 2.25 min; m/z = 438.28 [M+H]$^+$. HRMS: C$_{25}$H$_{29}$ClN$_3$O$_2$, 438.1948 (calcd.), 438.1997 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.38 (s, 9H), 1.77-1.84 (m, 3H), 2.04 (m, 1H), 2.13 (m, 1H), 2.34 (m, 1H), 3.03 (m, 1H), 3.51 (t, 1H, J = 12.6 Hz), 4.11-4.14 (dd, 1H, J = 12.0, 4.8 Hz), 5.33-5.36 (dd, 1H, J = 13.2, 4.8 Hz), 6.62 (s, 1H), 7.11 (d, 1H, J = 8.4 Hz), 7.24 (s, 1H), 7.44-7.46 (m, 2H), 7.50-7.51 (m, 2H), 7.67 (d, 1H, J = 7.2 Hz). ^{13}C NMR (150 MHz, CDCl$_3$): 17.8, 25.0, 26.5, 28.8, 38.3, 50.7, 51.2, 66.3, 128.6, 129.4, 130.7, 131.0, 131.2, 134.6, 134.7, 137.2, 137.4, 167.9, 168.9, 175.7.

Preparation of **1-acetyl-*N*-benzyl-7-chloro-5-(2-chlorophenyl)-2,3-dihydro-1*H*-benzo[e][1,4] diazepine-2-carboxamide (94h, Method E)**: The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 1:1) as yellow solid (12 mg, yield: 13% over two steps). HRMS: C$_{25}$H$_{22}$Cl$_2$N$_3$O$_2$, 466.1089 (calcd.), 466.1124 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.98 (s, 3H), 3.66 (t, 1H, J = 12.6 Hz), 4.21-4.24 (dd, 1H, J = 12.0, 4.8 Hz), 4.42 (m, 1H), 4.51 (m, 1H), 5.60-5.63 (dd, 1H, J = 13.2, 4.8 Hz), 6.98 (m, 1H), 7.09 (d, 1H, J = 8.4 Hz), 7.17 (m, 1H), 7.30-7.32 (m, 3H), 7.36-7.46 (m, 6H), 7.61 (d, 1H, J = 7.2 Hz). ^{13}C NMR (150 MHz, CDCl$_3$): 23.0, 43.4, 50.9, 66.2, 127.3, 127.5, 127.6, 127.9, 128.7, 130.3, 131.1, 131.3, 131.5, 131.7, 132.6, 134.8, 135.1, 137.1, 137.3, 138.1, 168.5, 169.6, 171.6.

3.4.2 Design of anchor-directed benzodiazepine scaffold

PPIs cannot be cost-efficiently tackled using conventional high-throughput screening methods. It is necessary to design new strategies that will maximize the chance for hit identification through a rationalization of the PPI inhibitor chemical space and the design of PPI-focused compound libraries.[322] Key characteristics of PPI inhibitors can then be revealed and highlight the importance of specific shapes and/or aromatic bonds, enabling the design of PPI inhibitors enriched focused libraries and cost-effective screening strategies.[323] It is possible to design compound libraries by refining the appropriate cores and periphery to create more focused compounds, with the increased likelihood of binding to a protein-protein interaction interface.

The development of new synthetic scaffolds is of uttermost importance for identifying new lead structures during the drug discovery process. Seven-membered 1,4-diazepine ring based drugs have been found repeatedly as the prototype of a privileged structure with a broad range of biological activities and applications in human medicine.[324] Over the last decades, 1,4-benzodiazepines have emerged as a particularly fascinating class of scaffolds in medicinal chemistry because they hit various classes of pharmacologically relevant targets such as GPCRs, ion channels and enzymes.[325] For example, anthramycin is a broad-spectrum antineoplastic antibiotic with low toxicity, which binds irreversibly to DNA and inhibits the synthesis of RNA and DNA.[326] Recently, BDZs have been widely investigated as potential medicinal agents.[327, 328] Therefore, preparation and evaluation of libraries based on privileged structures become an important part of the drug discovery process.

N-Boc-amino acids are ideal building blocks to introduce "anchor" fragments which can be incorporated into drug-like compounds via MCRs. To date, it has been shown that N-Boc-

amino acids were utilized to synthesize piperazines,[245, 329-331] 1,4-diazepines,[332] and other peptidomimetic scaffolds.[333] Herein, we designed a novel 1,4-benzodiazepine scaffold incorporated with "anchors" which can be accessed by the Ugi-4CR with N-Boc-amino acids.

First, we developed a synthetic method to allow rapid access to 1,4-benzodiazepines **96** in just two steps from Boc glycine. In the first step, aminophenylketones serve as an amine component for Ugi four-compponent reaction with Boc glycine, isocyanide, and aldehyde. The crude Ugi products were treated with TFA in the presence of DCE to produce 1,4-benzodiazepines in excellent yields (**Scheme 32**).
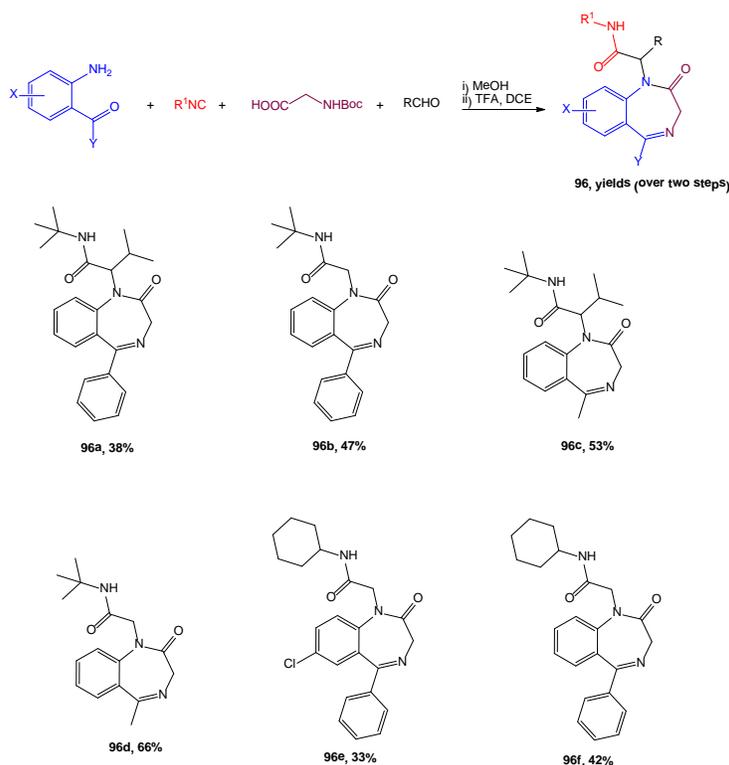
Scheme 32. Synthesis of 1,4-benzodiazepines **96**

Hence, we initiated to test the feasibility for the synthesis of "anchor" biased compound libraries. Phenylalanine, leucine, trptophan and tyrosine, which are abundant in the protein-protein interaction interface, were selected. N-Boc-amino acids were applied to the same

protocol for the synthesis of 1,4-benzodiazepines with variable aminophenylketones and isocyanides (**Scheme 33**). Compounds **97a-m** were isolated by chromatography in 22-69% yield over two steps.
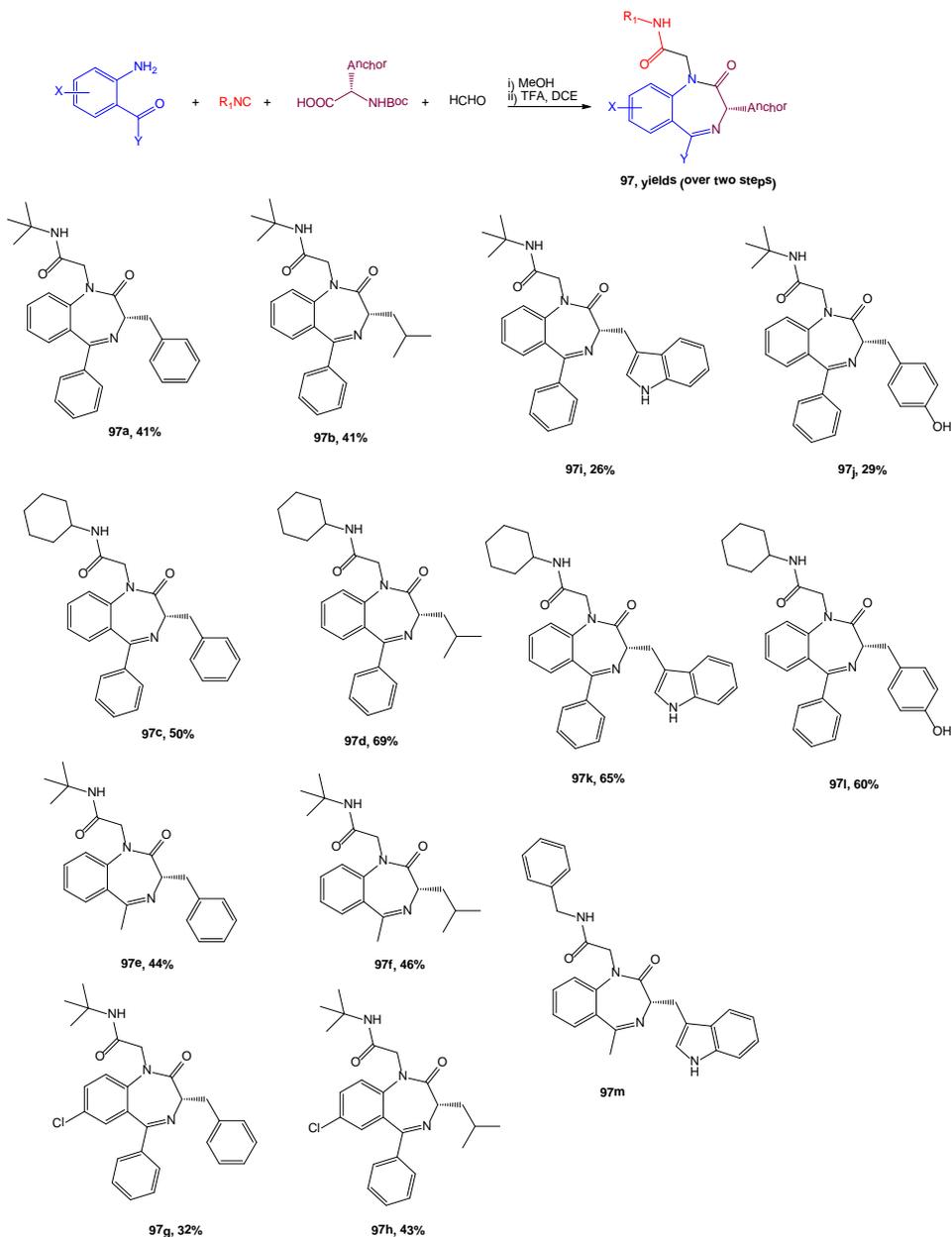
Scheme 33. Synthesis of 1,4-benzodiazepines **97**

In summary, we have designed a nove 1,4-benzodiazepine scaffold which allow to introduce "anchor" fragments at 3-position. A small focused "anchor" biased compound libraries

were obtained using glycine, phenylalanine, leucine, trptophan and tyrosine as the starting materials. This method has shown the advantages to efficiently generate diverse drug-like compounds incorporating a variety of "anchor" fragments, which are particularly interesting for screening campaigns against PPIs.

Materials and Methods

Representative procedure for the synthesis of 1,4-benzodiazepines:

Preparation of **_N_-tert-butyl-3-methyl-2-(2-oxo-5-phenyl-2,3-dihydro-1_H_-benzo[e][1,4] diazepin-1-yl)butanamide (96a)**: The mixture of 2-aminobenzophenone (0.2 mmol, 39.4 mg), _N_-Boc-glycine (0.2 mmol, 35.0 mg), _tert_-butyl isocyanide (0.2 mmol, 28.3 μL), formaldehyde (0.2 mmol, 22.8 μL) and 0.5 mL of methanol was stirring for 2 days under room temperature. After evaporation of the solvent, the residue was treated by 0.75 mL of DCE (10% TFA) was stirring under 40 $^{\circ}$C overnight. The residue was treated by triethylamine (150 μL), and the product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellowish solid (39 mg, yield: 38% over two steps). HPLC/MS: t_R = 12.04 min; m/z = 392.1 [M+H]$^+$. HRMS: $C_{24}H_{29}N_3O_2Na$, 414.2182 (calcd.), 414.2157 (found). ^{1}H NMR (600 MHz, CDCl$_3$, 2:1 mixture of rotamers): 0.49 (d, 3H, J = 6.6 Hz), 0.63 (d, 2H, J = 6.6 Hz), 0.93 (d, 2H, J = 6.6 Hz), 0.98 (d, 3H, J = 6.6 Hz), 1.39 (s, 9H), 1.40 (s, 5H), 2.26 (m, 1H), 2.92 (m, 1H), 3.55 (d, 1H, J = 11.4 Hz), 3.83-3.85 (m, 2H), 4.43 (d, 1H, J = 11.4 Hz), 4.72 (d, 1H, J = 10.6 Hz), 4.82 (d, 1H, J = 11.4 Hz), 6.88 (s, 1H), 7.23-7.32 (m, 3H), 7.39-7.59 (m, 7H), 7.64 (m, 2H), 7.76 (d, 1H, J = 8.4 Hz), 8.02 (s, 1H), 8.30 (d, 1H, J = 8.4 Hz). ^{13}C NMR (150 MHz, CDCl$_3$, 2:1 mixture of rotamers): 18.9, 19.68, 19.73, 20.0, 25.8, 27.6, 28.6, 51.0, 51.4, 57.1, 58.3, 123.9,

124.7, 125.0, 125.2, 128.4, 129.0, 129.4, 129.6, 129.7, 130.2, 130.5, 130.6, 131.0, 132.1, 138.1, 138.4, 140.4, 145.8, 169.9, 170.6, 170.7, 171.1, 171.8, 172.5.

Preparation of **N-*tert*-butyl-2-(2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-1-yl)acetamide (96b)**: The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as white solid (29 mg, yield: 47% over two steps). SFC/MS: t_R = 2.60 min; m/z = 348.24 [M-H]$^-$. HRMS: $C_{21}H_{23}N_3O_2Na$, 372.1688 (calcd.), 372.1709 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.28 (s, 9H), 3.89 (d, 1H, J = 10.8 Hz), 4.16 (d, 1H, J = 15.6 Hz), 4.53 (d, 1H, J = 15.6 Hz), 4.85 (d, 1H, J = 10.8 Hz), 6.18 (s, 1H), 7.24-7.26 (m, 1H), 7.32-7.34 (m, 1H), 7.40-7.43 (m, 2H), 7.47-7.49 (m, 1H), 7.57-7.60 (m, 1H), 7.63-7.64 (m, 2H), 7.68-7.70 (m, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 28.5, 51.5, 53.4, 56.7, 122.4, 124.8, 128.3, 128.8, 129.6, 130.4, 130.7, 132.0, 138.6, 143.0, 167.7, 170.3, 170.8.

Preparation of **N-*tert*-butyl-3-methyl-2-(5-methyl-2-oxo-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-1-yl)butanamide (96c)**: The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellow solid (35 mg, yield: 53% over two steps). SFC/MS: t_R = 1.62 min; m/z = 330.28 [M+H]$^+$. HRMS: $C_{19}H_{27}N_3O_2Na$, 352.2001 (calcd.), 352.1990 (found). ^{1}H NMR (600 MHz, CDCl$_3$, 2:1 mixture of rotamers): 0.41 (d, 3H, J = 6.0 Hz), 0.73 (d, 2H, J = 6.6 Hz), 0.95-0.98 (m, 5H), 1.39 (s, 14H), 2.13 (m, 1H), 2.47-2.48 (m, 5H), 2.94 (m, 1H), 3.48 (m, 1H), 3.66-3.68 (m, 2H), 4.41 (d, 1H, J = 11.4 Hz), 4.46 (d, 1H, J = 11.4 Hz), 4.55 (d, 1H, J = 11.4 Hz), 6.88 (s, 1H), 7.30-7.32 (m, 2H), 7.47-7.54 (m, 3H), 7.67 (d, 1H, J = 7.8 Hz), 7.94 (s, 1H), 8.15 (d, 1H, J = 8.4 Hz). ^{13}C NMR (150 MHz, CDCl$_3$, 2:1 mixture of rotamers): 18.4, 19.3, 19.7, 20.1, 25.1, 25.2, 25.7, 27.6, 28.6, 45.7, 50.9, 51.3, 56.5, 57.6, 65.7, 79.4, 124.0, 124.6, 125.6, 125.7, 126.8, 127.0, 130.3, 130.6, 131.7, 132.0, 138.5, 143.9, 160.9, 170.3, 170.4, 170.5, 171.9, 172.5.

Preparation of **N-*tert*-butyl-2-(5-methyl-2-oxo-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-1-yl)acetamide (96d)**: The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellowish solid (38 mg, yield: 66% over two steps). HRMS: $C_{16}H_{21}N_3O_2Na$, 310.1531 (calcd.), 310.1562 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.35 (s, 9H), 2.51 (s, 3H), 3.70 (d, 1H, *J* = 10.8 Hz), 4.00 (d, 1H, *J* = 15.6 Hz), 4.53-4.59 (m, 2H), 6.24 (s, 1H), 7.28-7.31 (m, 1H), 7.51-7.55 (m, 2H), 7.61 (m, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 25.7, 28.6, 51.5, 53.6, 56.1, 122.5, 125.3, 127.5, 130.2, 131.7, 141.3, 167.7, 170.1, 170.2.

Preparation of **2-(7-chloro-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-1-yl)-*N*-cyclohexylacetamide (96e)**: The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellowish solid (27 mg, yield: 33% over two steps). SFC/MS: t_R = 3.01 min; *m/z* = 410.23 [M+H]$^+$. HRMS: $C_{23}H_{25}ClN_3O_2$, 410.1635 (calcd.), 410.1677 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.03-1.10 (m, 3H), 1.26-1.31 (m, 3H), 1.59-1.62 (m, 2H), 1.77-1.86 (m, 2H), 3.70 (m, 1H), 3.86 (ABd, 1H, *J* = 10.8 Hz), 4.18 (ABd, 1H, *J* = 15.0 Hz), 4.53 (ABd, 1H, *J* = 15.6 Hz), 4.86 (ABd, 1H, *J* = 10.8 Hz), 6.16 (m, 1H), 7.29 (m, 1H), 7.42-7.44 (m, 2H), 7.49-7.53 (m, 2H), 7.61-7.63 (m, 2H), 7.70 (m, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 24.6, 25.4, 32.6, 48.6, 52.6, 56.7, 124.1, 128.5, 129.5, 129.7, 130.2, 130.3, 130.9, 131.9, 138.0, 141.6, 167.1, 169.5, 169.8.

Preparation of **N-cyclohexyl-2-(2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-1-yl)acetamide (96f)**: The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellowish solid (32 mg, yield: 42% over two steps). SFC/MS: t_R = 4.20 min; *m/z* = 377.27 [M+H]$^+$. HRMS: $C_{23}H_{26}N_3O_2$, 376.2025 (calcd.), 376.2064 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.96 (m, 1H), 1.06-1.10 (m, 2H), 1.26-1.32 (m, 2H), 1.54-1.63 (m, 3H), 1.74-1.76 (m, 1H), 1.84-1.86 (m, 1H), 3.72 (m, 1H), 3.89 (ABd, 1H, *J* = 10.2 Hz), 4.30

(ABd, 1H, J = 15.6 Hz), 4.58 (ABd, 1H, J = 15.6 Hz), 4.87 (ABd, 1H, J = 10.8 Hz), 6.16 (m, 1H), 7.25-7.27 (m, 1H), 7.34-7.36 (m, 1H), 7.41-7.44 (m, 2H), 7.48-7.51 (m, 1H), 7.58-7.61 (m, 1H), 7.65 (m, 1H), 7.69 (m, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 24.6, 25.4, 32.6, 48.5, 52.6, 56.7, 122.4, 124.9, 128.3, 128.9, 129.6, 130.4, 130.7, 131.9, 138.5, 142.9, 167.4, 170.4, 170.7.

Preparation of **(S)-2-(3-benzyl-2-oxo-5-phenyl-2,3-dihydro-1H-benzo[e][1,4]diazepin-1-yl)-N-tert-butylacetamide (97a)**: the product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellow solid (36 mg, 41% over two steps). SFC/MS: t_R = 2.89 min; m/z = 438.31 [M-H]$^-$. HRMS: C$_{28}$H$_{29}$N$_3$O$_2$Na, 462.2157 (calcd.), 462.2180 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.23 (s, 9H), 3.58 (dd, 1H, J = 7.2, 14.4 Hz), 3.71 (dd, 1H, J = 6.6, 13.8 Hz), 3.91 (t, 1H, J = 7.2 Hz), 4.16 (ABd, 1H, J = 15 Hz), 4.58 (ABd, 1H, J = 15 Hz), 6.12 (s, 1H), 7.20-7.24 (m, 2H), 7.29-7.31 (m, 3H), 7.37-7.42 (m, 4H), 7.47-7.57 (m, 1H), 7.58-7.61 (m, 3H), 7.66-7.68 (m, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 28.4, 38.0, 51.4, 51.9, 65.1, 122.5, 124.7, 126.2, 128.26, 128.29, 129.2, 129.78, 129.82, 130.3, 130.6, 132.0, 138.6, 139.1, 142.6, 167.8, 168.8, 170.5.

Preparation of **(S)-N-tert-butyl-2-(3-isobutyl-2-oxo-5-phenyl-2,3-dihydro-1H-benzo[e][1,4]diazepin-1-yl)acetamide (97b)**: The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellow solid (33 mg, 41% over two steps). SFC/MS: t_R = 2.44 min; m/z = 404.31 [M-H]$^-$. HRMS: C$_{25}$H$_{31}$N$_3$O$_2$Na, 428.2314 (calcd.), 428.2302 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.84 (d, 3H, J = 6.6 Hz), 1.01 (d, 3H, J = 6.6 Hz), 1.26 (s, 9H), 1.93-1.99 (m, 1H), 2.02-2.07 (m, 1H), 2.29-2.34 (m, 1H), 3.69-3.71 (m, 1H), 4.19 (ABd, 1H, J = 15.0 Hz), 4.54 (ABd, 1H, J = 15.0 Hz), 6.13 (s, 1H), 7.24-7.27 (m, 1H), 7.34-7.36 (m, 1H), 7.39-7.50 (m, 2H), 7.59-7.63 (m, 4H), 7.70-7.71 (m, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 22.0, 23.4, 24.8,

28.5, 40.0, 51.4, 53.7, 61.6, 122.4, 124.7, 128.3, 129.3, 129.7, 130.1, 130.5, 131.9, 138.6, 142.7, 167.9, 168.8, 171.1.

Preparation of **(*S*)-2-(3-benzyl-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-1-yl)-*N*-cyclohexylacetamide (97c)**: The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellow solid (66 mg, 50% over two steps). SFC/MS: t_R = 3.21 min; m/z = 464.32 [M-H]$^-$. HRMS: $C_{30}H_{31}N_3O_2Na$, 488.2314 (calcd.), 488.2334 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.92-0.94 (m, 2H), 1.09-1.10 (m, 1H), 1.27-1.28 (m, 2H), 1.61 (m, 3H), 1.75 9(m, 2H), 3.60 (dd, 1H, J = 7.2 Hz), 3.65-3.72 (m, 2H), 3.92 (t, 1H, J = 6.6 Hz), 4.27 (ABd, 1H, J = 15.6 Hz), 4.64 (ABd, 1H, J = 15 Hz), 6.198 (s, 1H), 7.20-7.24 (m, 2H), 7.29-7.31 (m, 3H), 7.37-7.42 (m, 4H), 7.46-7.49 (m, 1H), 7.56-7.58 (m, 1H), 7.61-7.62 (m, 2H), 7.65-7.67 (m, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 24.7, 25.4, 32.5, 32.6, 38.0, 48.4, 52.9, 65.1, 122.4, 124.8, 126.2, 128.27, 128.30, 129.2, 129.78, 129.84, 130.3, 130.6, 132.0, 138.5, 139.0, 142.5, 167.5, 168.8, 170.5.

Preparation of **(*S*)-*N*-cyclohexyl-2-(3-isobutyl-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-1-yl)acetamide (97d)**: The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellow solid (60 mg, 69% over two steps). SFC/MS: t_R = 2.72 min; m/z = 430.32 [M-H]$^-$. HRMS: $C_{27}H_{33}N_3O_2Na$, 454.2470 (calcd.), 454.2457 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.83 (d, 3H, J = 6.6 Hz), 0.94-1.05 (m, 6H), 1.23-1.29 (m, 2H), 1,51-1.63 (m, 3H), 1.71 (m, 1H), 1.81 (m, 1H), 1.95-2.04 (m, 2H), 2.30-2.33 (m, 1H), 3.69-3.71 (m, 2H), 4.30 (ABd, 1H, J = 15.6 Hz), 4.58 (ABd, 1H, J = 15 Hz), 6.26 (s, 1H), 7.23-7.25 (m, 1H), 7.33-7.35 (m, 1H), 7.38-7.40 (m, 2H), 7.44-7.47 (m, 1H), 7.89-7.59 (m, 1H) 7.61-7.63 (m, 2H), 7.69-7.70 (m, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 22.0, 23.4, 24.6, 24.7, 24.8, 25.4, 32.5, 32.6,

40.0, 48.4, 52.7, 61.6, 122.4, 124.7, 128.3, 129.3, 129.7, 130.0, 130.5, 131.9, 138.6, 142.6, 167.6, 168.7, 171.1.

Preparation of **(S)-2-(3-benzyl-5-methyl-2-oxo-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-1-yl)-*N-tert*-butylacetamide (97e)**: The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellow solid (33 mg, 44% over two steps). SFC/MS: t_R = 2.58 min; m/z = 376.29 [M-H]⁻. HRMS: $C_{23}H_{28}N_3O_2$, 378.2182 (calcd.), 378.2148 (found). ^{1}H NMR (600 MHz, CDCl₃): 1.267 (s, 9H), 2.51 (s, 3H), 3.31-3.33 (m, 1H), 3.72-3.77 (m, 2H), 3.94 (ABd, 1H, J = 15 Hz), 4.63 (ABd, 1H, J = 15 Hz), 6.198 (s, 1H), 7.16-7.18 (m, 1H), 7.23-7.25 (m, 2H), 7.28-7.30 (m, 3H), 7.51-7.55 (m, 2H), 7.58-7.59 (m, 1H). ^{13}C NMR (150 MHz, CDCl₃): 25.6, 28.5, 37.8, 51.3, 54.1, 64.5, 122.6, 125.3, 126.2, 127.4, 128.3, 129.6, 130.5, 131.7, 138.9, 140.8, 167.9, 168.2, 169.9.

Preparation of **(S)-*N-tert*-butyl-2-(3-isobutyl-5-methyl-2-oxo-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-1-yl)acetamide (97f)**: The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellow solid (32 mg, 46% over two steps). SFC/MS: t_R = 2.16 min; m/z = 342.30 [M-H]⁻. HRMS: $C_{20}H_{30}N_3O_2$, 344.2338 (calcd.), 344.2355 (found). ^{1}H NMR (600 MHz, CDCl₃): 0.80 (d, 3H, J = 6.6 Hz), 0.92 (d, 3H, J = 7.2 Hz), 1.34 (s, 9H), 1.77-1.82 (m, 1H), 2.04 (t, 2H, J = 6.9 Hz), 2.49 (s, 3H), 3.51 (t, 1H, J = 7.2 Hz), 4.00 (ABd, 1H, J = 15 Hz), 4.58 (ABd, 1H, J = 15 Hz), 6.232 (s, 1H), 7.29-7.31 (m, 1H), 7.52-7.56 (m, 2H), 7.63-7.65 (m, 1H). ^{13}C NMR (150 MHz, CDCl₃): 22.1, 23.1, 24.7, 25.5, 28.6, 40.0, 51.4, 53.9, 60.9, 122.5, 125.2, 127.2, 130.6, 131.6, 141.0, 167.96, 167.99, 170.7.

Preparation of **(S)-2-(3-benzyl-7-chloro-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-1-yl)-*N-tert*-butylacetamide (97g)**: the product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellow solid (30 mg, 32% over two

steps). SFC/MS: t_R = 3.06 min; m/z = 472.27 [M-H]⁻. HRMS: $C_{28}H_{29}ClN_3O_2$, 474.1948 (calcd.), 474.1948 (found). ¹H NMR (600 MHz, CDCl₃): 1.26 (s, 9H), 3.57 (dd, 1H, J = 7.2, 13.8 Hz), 3.68 (dd, 1H, J = 6, 13.8Hz), 3.89 (t, 1H, J = 6.6 Hz), 4.08 (ABd, 1H, 15 Hz), 4.55 (ABd, 1H, 15 Hz), 6.12 (s, 1H), 7.21-7.24 (m, 1H), 7.26 (s, 1H), 7.28-7.32 (m, 2H), 7.36-7.37 (m, 2H), 7.42-7.44 (m, 2H), 7.48-7.53 (m, 2H), 7.58-7.59 (m, 2H), 7.69-7.71 (m, 1H). ¹³C NMR (150 MHz, CDCl₃): 28.5, 37.9, 51.5, 53.9, 65.1, 124.2, 126.3, 128.3, 128.5, 129.6, 129.7, 129.8, 130.2, 130.4, 130.9, 132.0, 138.0, 138.8, 141.2, 167.5, 167.6, 170.0.

Preparation of **(S)-N-tert-butyl-2-(7-chloro-3-isobutyl-2-oxo-5-phenyl-2,3-dihydro-1H-benzo [e][1,4]diazepin-1-yl)acetamide (97h):** The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellow solid (38 mg, 43% over two steps). SFC/MS: t_R = 2.64 min; m/z = 438.24 [M-H]⁻. HRMS: $C_{25}H_{31}ClN_3O_2$, 440.2105 (calcd.), 440.2103 (found). ¹H NMR (600 MHz, CDCl₃): 0.85 (d, 3H, J = 6.6 Hz), 1.02 (d, 3H, J = 6.6 Hz), 1.28 (s, 9H), 1.93-1.97 (m, 1H), 2.00-2.05 (m, 1H), 2.28-2.33 (m, 1H), 3.68 (dd, 1H, J = 5.1, 8.7 Hz), 4.10 (ABd, 1H, J = 15 Hz), 4.51 (ABd, 1H, J = 15 Hz), 6.17 (s, 1H), 7.31 (m, 1H), 7.41-7.43 (m, 2H), 7.47-7.50 (m, 1H), 7.53-7.55 (m, 1H), 7.60-7.61 (m, 2H), 7.73-7.75 (m, 1H). ¹³C NMR (150 MHz, CDCl₃): 22.0, 23.4, 24.8, 28.5, 40.0, 51.5, 53.7, 61.7, 124.2, 128.5, 129.4, 129.6, 130.1, 130.5, 130.8, 132.0, 138.1, 141.4, 167.5, 167.6, 170.6.

Preparation of **(S)-2-(3-((1H-indol-3-yl)methyl)-2-oxo-5-phenyl-2,3-dihydro-1H-benzo[e] [1,4]diazepin-1-yl)-N-tert-butylacetamide (97i)**: The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellow solid (25 mg, 26% over two steps). SFC/MS: t_R = 4.28 min; m/z = 477.32 [M-H]⁻. HRMS: $C_{30}H_{31}N_4O_2$, 479.2447 (calcd.), 479.2451 (found). ¹H NMR (600 MHz, CDCl₃): 1.22 (s, 9H), 3.69 (dd, 1H, J = 6.6, 14.4Hz), 3.87-3.90 (m, 1H), 3.94 (t, 1H, J = 6.6 Hz), 4.22 (ABd, 1H, J = 15 Hz), 4.57 (ABd, 1H, J = 15

Hz), 6.07(s, 1H), 7.10-7.12 (m, 1H), 7.17-7.21 (m, 2H), 7.29-7.30 (m, 1H), 7.35-7.36 (m, 1H), 7.40-7.42 (m, 2H), 7.47-7.48 (m, 1H), 7.53-7.56 (m, 1H), 7.60-7.64 (m, 3H), 7.68-7.70 (m, 1H), 8.09 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 27.3, 28.4, 51.4, 53.7, 64.5, 111.0, 113.0, 119.1, 119.2, 121.8, 122.4, 123.3, 124.7, 127.8, 128.3, 129.3, 129.8, 130.2, 130.6, 131.9, 136.0, 138.6, 142.5, 167.8, 168.7, 170.8.

Preparation of **(*S*)-*N*-*tert*-butyl-2-(3-(4-hydroxybenzyl)-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-1-yl)acetamide (97j)**: The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellow solid (26 mg, 29% over two steps). HPLC/MS: t_R = 10.41 min; m/z = 456.0 [M+H]$^+$. HRMS: C$_{28}$H$_{30}$N$_3$O$_3$, 456.2287 (calcd.), 456.2286 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 1.23 (s, 9H), 3.48 (dd, 1H, J = 7.2, 15 Hz), 3.61 (dd, 1H, J = 6.3, 14.1 Hz), 3.83 (t, 1H, J = 6.9 Hz), 4.19 (ABd, 1H, J = 15 Hz), 4.56 (ABd, 1H, J = 15.6 Hz), 6.10 (s, 1H), 6.75-6.76 (m, 2H), 7.20-7.22 (m, 3H), 7.29-7.30 (m, 1H), 7.39-7.41 (m, 2H), 7.46-7.48 (m, 1H), 7.54-7.57 (m, 1H), 7.59-7.60 (m, 2H), 7.62-7.63 (m, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 29.4, 37.0, 51.5, 53.7, 65.3, 115.1, 122.4, 124.8, 128.3, 129.2, 129.8, 130.3, 130.6, 130.7, 130.9, 132.0, 138.5, 142.5, 154.3, 167.9, 168.9, 170.5.

Preparation of **(*S*)-2-(3-((1*H*-indol-3-yl)methyl)-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-1-yl)-*N*-cyclohexylacetamide (97k)**: The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellow solid (66 mg, 65% over two steps). SFC/MS: t_R = 4.54 min; m/z = 503.33 [M-H]$^-$. HRMS: C$_{32}$H$_{33}$N$_4$O$_2$, 505.2604 (calcd.), 505.2605 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.86-0.88 (m, 2H), 1.02-1.07 (m, 1H), 1.16-1.30 (m, 3H), 1.51-1.61 (m, 2H), 1.72-1.74 (m, 2H), 3.67-3.71 (m, 2H), 3.86-3.87 (m, 1H), 3.96 (t, 1H, J = 7.2 Hz), 4.34 (ABd, 1H, J = 15.6 Hz), 4.62 (ABd, 1H, J = 15.6 Hz), 6.19 (s, 1H), 7.10-7.11 (m, 1H), 7.15-7.19 (m, 3H), 7.27-7.28 (m, 1H), 7.32-7.33 (m, 1H), 7.39-7.42 (m, 2H), 7.46-

7.47 (m, 1H), 7.52-7.53 (m, 1H), 7.57-7.58 (m, 1H), 7.64-7.65 (m, 2H), 7.68-7.70 (m, 1H), 8.42 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 24.6, 24.7, 24.8, 25.4, 27.4, 32.4, 32.5, 33.0, 48.5, 52.7, 64.5, 111.1, 112.7, 119.0, 119.1, 121.7, 122.3, 123.4, 124.7, 127.8, 128.3, 129.3, 129.8, 130.3, 130.6, 131.9, 136.1, 138.5, 142.4, 167.6, 168.8, 170.8.

Preparation of **(*S*)-*N*-cyclohexyl-2-(3-(4-hydroxybenzyl)-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-1-yl)acetamide (97l)**: The product was isolated by silica gel chromatography (petroleum ether/ethyl acetate, 2:1) as yellow solid (58 mg, 60% over two steps). SFC/MS: t_R = 4.50 min; *m/z* = 480.32 [M-H]$^-$. HRMS: C$_{30}$H$_{32}$N$_3$O$_3$, 482.2444 (calcd.), 482.2444 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 0.90-0.94 (m, 2H), 1.06 (m, 1H), 1.20-1.23 (m, 2H), 1.53-1.58 (m, 3H), 1.69-1.74 (m, 2H), 3.46-3.49 (m, 1H), 3.55-3.59 (m, 1H), 3.65-3.66 (m, 1H), 3.83 (t, 1H, *J* = 6.6 Hz), 4.31 (ABd, 1H, *J* = 15.6 Hz), 4.60 (ABd, 1H, *J* = 15.6 Hz), 6.24-6.25 (m, 1H), 6.74-6.75 (m, 2H), 7.15-7.20 (m, 3H), 7.26-7.28 (m, 1H), 7.37-7.42 (m, 2H), 7.44-7.46 (m, 1H), 7.51-7.53 (m, 1H), 7.58-7.61 (m, 3H). ^{13}C NMR (150 MHz, CDCl$_3$): 24.6, 25.3, 32.44, 32.49, 36.9, 48.6, 52.7, 65.3, 115.3, 122.3, 124.8, 128.3, 129.2, 129.8, 130.1, 130.3, 130.7, 130.8, 132.0, 138.4, 142.3, 154.9, 167.8, 169.0, 170.6.

Preparation of **(*S*)-2-(3-((1*H*-indol-3-yl)methyl)-5-methyl-2-oxo-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-1-yl)-*N*-benzylacetamide (97m)**: Yellowish solid, 20 mg (22%). SFC/MS: t_R = 4.59 min; *m/z* = 451.26 [M+H]$^+$. HRMS: C$_{28}$H$_{26}$N$_4$O$_2$Na, 473.1953 (calcd.), 473.1957 (found). ^{1}H NMR (600 MHz, CDCl$_3$): 2.44 (s, 3H), 3.42 (dd, 1H, *J* = 5.4, 14.4 Hz), 3.74-3.76 (m, 1H), 3.83-3.86 (m, 1H), 4.16 (ABd, 1H, *J* = 15.6 Hz), 4.30 (dd, 1H, *J* = 5.4, 14.4 Hz), 4.53 (dd, 1H, *J* = 6.0, 15.0 Hz), 4.84 (ABd, 1H, *J* = 15.6 Hz), 6.58 (br.s, 1H), 6.93 (m, 1H), 7.04-7.06 (m, 1H), 7.13-7.16 (m, 1H), 7.21-7.23 (m, 2H), 7.27 (m, 1H), 7.35-7.36 (m, 2H), 7.49-7.53 (m, 2H), 7.57 (m, 1H), 7.60 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$): 25.4, 27.0, 43.6, 52.9, 63.9, 111.0, 112.5,

118.7, 119.1, 121.8, 122.0, 122.3, 123.4, 125.3, 127.5, 127.8, 128.8, 130.6, 131.7, 137.9, 140.6, 168.1, 168.7, 170.4.

4.0   MAJOR FINDINGS AND IMPLICATIONS

4.1   SUMMARY

PPIs represent prospective targets for next generation of drugs that address unmet medical needs, such as cancer, diabetes and Alzheimer's disease. Unlike the traditional drug targets, PPIs remain challenges in terms of drug discovery. We designed a novel, complementary and transformative approach for the rational design of PPI inhibitors, which allows fast generation of potential lead compounds. This method is based on a tight interplay of structural biology information, the "anchor" concept (Anchor Database), robust synthesis and screening process. On the other hand, MCR strategies have been developed for the design and synthesis of novel scaffolds as potential PPI inhibitor templates. Anchor-biased compound libraries have been generated for pharmacophore-based virtual screening platform (AnchorQuery) against PPIs. This innovative approach and handful technologies have shown to accelerate the discovery of small molecules as PPI inhibitors, which can be useful as the starting point for drug discovery or molecular probes for regulating PPI pathways.

One approach to develop novel anti-cancer agents is the inhibition of the interaction between the tumor suppressor p53 and the oncogene products (Mdm2, Mdm4). Several new scaffolds as the inhibitors of p53-Mdm2 interaction were efficiently discovered by applying this integrative approach. Medicinal chemistry strategies (e.g., the substitution of fluorine,

modification of side chains) were explored in order to optimize the pharmacological activities. Advantages of the approach include high hit rates, less attrition based on the parallel discovery of multiple scaffolds, and convenient SAR study using efficient MCRs. The p53-Mdm2 inhibitors were identified by biochemical assays, co-crystallization and NCI-60 cell-based assays. The potential anticancer drug candidates based on pyrazole scaffold are undergoing pre-clinical investigations (e.g., aqueous solubility, in vitro metabolism, in vivo animal model studies).

The development of new synthetic scaffolds is of uttermost importance for identifying new chemotypes targeting PPIs. MCRs were applied as an efficient chemical synthesis toolbox that yields highly diverse and complex, drug-like and screening-ready products. The design and synthesis of two new scaffolds of TDZ have been reported. Consequently, a series of four 1,4-benzodiazepine scaffolds have been synthesized using MCR strategies. This approach possesses novel hybrid peptidomimetic scaffolds with well-defined diversity, and some compounds have shown to inhibit the p53-Mdm2 and other protein-protein interaction targets. These synthetic methodologies can be easily adapted for high throughput chemistry or medicinal chemistry in order to generate high-dimensional combinatorial libraries for screening purpose.

Due to in silico and computational advances, chemoinformatic and virtual screening tools have been used to identify promising small molecules of greater importance in drug discovery. I have generated random virtual libraries of new scaffolds and evaluated the drug-like properties and chemical space distributions. Meanwhile, anchor derived libraries can been generated in order to discover novel chemotypes targeting PPIs. These libraries are available for pharmacophore-based virtual screening platform AnchorQuery. So far, virtual libraries of >20 million compounds amenable to target any PPI with a tryptophan, tyrosine, phenylalanine,

leucine or valine anchor have been generated using 23 MCR chemistries and a curated set of starting materials.

4.2   FUTURE DIRECTIONS

4.2.1   Directions for short-term study

From NMR studies on Mdm2 fragments bound to N-terminal p53 peptides, it was suggested that Mdm2 is conformationally flexible, and subject to allosteric regulation upon substrate binding.[334] The structure of free Mdm2 N-terminal domain reveals that the more open conformation of the binding cleft of Mdm2 observed in structures of complexes with small molecules and peptides is a more suitable one for ligand discovery and optimisation.[335] It is worthwhile to use the small molecules with known binding mode to probe the flexible binding pocket of Mdm2, in order to reveal the potential new scaffolds for ligand design. We focus on the optimization of a peptidomimetic scaffold as p53-Mdm2 inhibitors, which can be easily accessed by Ugi MCR, and the binding model was established according to cocrystallization in **Chapter 2.3**.

In an attempt to optimize the amine component of p53-Mdm2 inhibitors, benzyl amine derivatives were used for the synthesis of Ugi derived scaffold (**Scheme 34**). The substituted benzylamines **98a-c** were obtained from commercial sources or prepared from the coupling of p-hydroxy benzyl amine with the corresponding benzyl bromides.[336] The ester compounds **99a-c** were synthesized by the Ugi-4CR, which were then saponified to give the corresponding acid

compounds **100a-c**, since 2-carboxylic acid of the indole ring is known to improve the binding affinity with Mdm2.
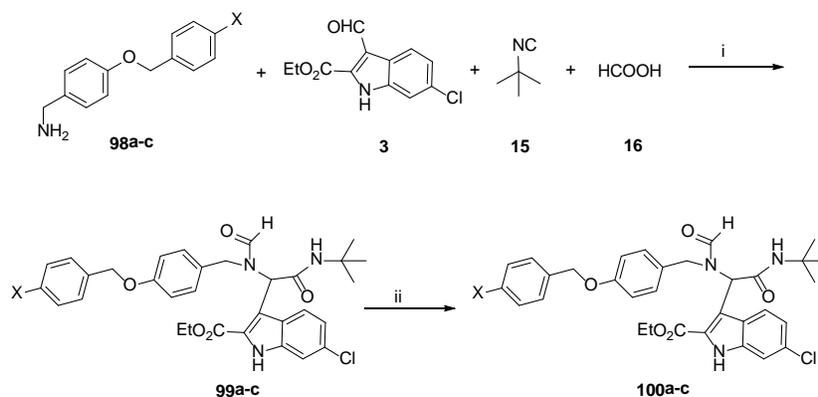
Scheme 34. Synthesis of p53-Mdm2 inhibitors. Conditions: (i) MeOH, r.t.; (ii) LiOH, EtOH/H$_2$O (1:1), r.t.

The binding constants (Ki) of the compounds **99** and **100** to Mdm2 were measured by FP assay (**Table 17**). Compounds **100a-b** are more potent than the parent compound **9r**, indicating that the binding pocket of Mdm2 is flexible if compounds **100a-b** have the similar binding pose with compound **9l** shown in **Figure 13** (**Chapter 2.3.1**). However, compound **100c** loses the binding with Mdm2 probably because the nitro group is not tolerant with the pocket.

Table 17. Inhibition constants [μM] of p53-Mdm2 inhibitors.

entry	X	Ki (**99**)	Ki (**100**)
a	Cl	Fluorescent	0.6
b	H	Fluorescent	4.9
c	NO$_2$	n.i.	Low interaction

| | | 11 |
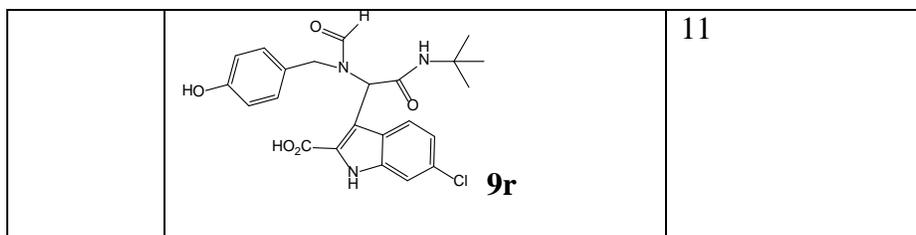
[a] Measured by fluorescent polarization assay.

N-terminal lid of Mdm2 has been implicated in p53 regulation; however, due to its flexible nature, limited data are available concerning its role in ligand binding.[337] NMR study has shown that Mdm2 residues 16-24 form a lid that closes over the p53-binding site, in addition to Mdm2 residues 25-109 that form the well ordered p53-binding domain that was observed in the p52-Mdm2 complex.[338] Recent studies reveal that apo-Mdm2 predominantly populates the closed state in which the lid is associated with the p53-binding cleft, whereas the p53-bound Mdm2 exclusively populates the open state in which the lid is highly flexible.[339] Unlike p53 binding, small molecule inhibitor nutlin-3 binds to the cleft essentially without perturbing the closed lid state.

Fortunately, compound **100a** was co-crystalized with Mdm2, shown in **Figure 49**. It has the similar binding pose with compound **9l** shown in **Figure 13** (**Chapter 2.3.1**): the indole fragment replaces the Trp23, and tert-butyl group occupies the Phe19 binding pocket. Suprisingly, a new pocket adjacent the Leu26 binding site is formed to accommodate the longer side chain. His96 is no longer parallel to the phenyl ring. However, the longer side chain seems to form hydrophobic interaction with the newly formed deep pocket, which is not shown in previously reported Mdm2 structures. This cocrystal structure provides a new avenue for the experimental and virtual screening of therapeutic inhibitors that target the p53-Mdm2 interaction.

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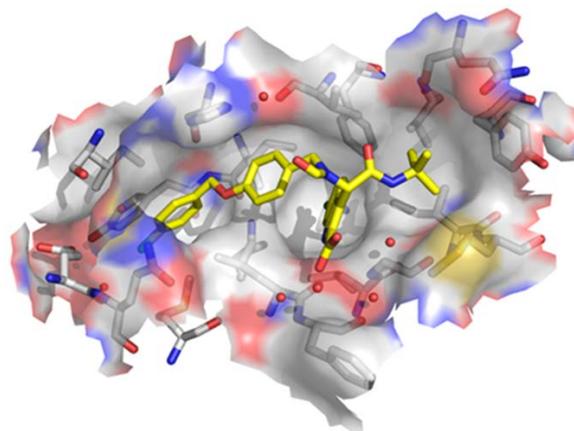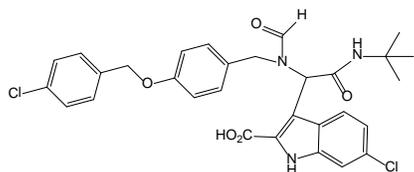
Figure 49. Cocrystal Structure of **100a**-Mdm2 complex reveals a new binding pocket for small molecules

(resolution: 2.0 Å).

Similarly, we also intended to optimize the isocyanide component of Ugi-derived scaffold as p53-Mdm2 inhibitors in order to test the flexibility of Phe19 binding pocket. Inspired by the most potent compound identified in **Chapter 2.3.2**, 2-isocyano-2,4,4-trimethylpentane was used for the Ugi-4CR (**Scheme 35**). Compound **104** was obtained after saponifiaction of compound **103** without further purification.
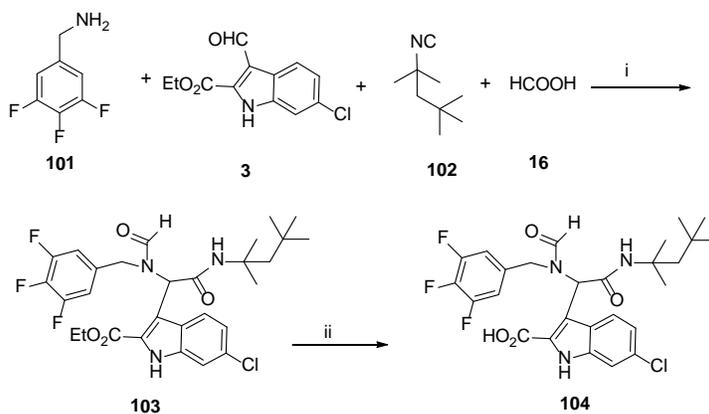
Scheme 35. Synthesis of p53-Mdm2 inhibitors via Ugi-4CR. Conditions: (i) MeOH, r.t.; (ii) LiOH, EtOH/H_2O

(1:1), r.t.

The Ki value of compound **104** to Mdm2 was measured by FP assay (**Figure 50**). Compound **104** has comparable binding affinity to Mdm2, indicating that the Phe19 binding pocket is also flexible if compound **104** has the similar binding pose with compound **18e** shown in **Figure 15** (**Chapter 2.3.2**). This also suggests that there is plenty of room for optimization and further design of revolutionary p53-Mdm2 inhibitors.

18m: Ki = 0.13 µM (Mdm2)   **104**: Ki = 0.2 µM (Mdm2)

Figure 50. Inhibition constants [µM] measured by FP assay

Materials and Methods

General procedure for the synthesis of compounds 99:

Preparation of **ethyl 3-(2-(*tert*-butylamino)-1-(*N*-(4-(4-chlorobenzyloxy)benzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (99a):** The mixture of ethyl 6-chloro-3-formyl-1*H*-indole-2-carboxylate (0.4 mmol, 100.4 mg), (4-(4-chlorobenzyloxy)phenyl)methanamine (0.4 mmol, 100 mg), *tert*-butyl isocyanide (0.4 mmol, 46 µL), formic acid (0.4 mmol, 16.0 µL) in 1 mL of methanol was stirring under RT for 5 days. The product was purified by chromatography on silica gel (petroleum ether/ethyl acetate, 1:1) as white solid (120 mg, yield: 49%). SFC/MS: t_R = 3.96 min; m/z = 608.31 [M+H]$^+$. HRMS: $C_{32}H_{33}N_3O_5Cl_2Na$, 632.1695 (calcd.), 632.1724 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.19 (s, 9H), 1.28 (s, 6H), 1.33-1.36 (m, 6H), 4.22-4.25 (m, 1H), 4.29-4.38 (m, 4H), 4.37 (m, 1H), 4.49 (ABd,

1H, J = 16.2 Hz), 4.76 (ABd, 1H, J = 15.0 Hz), 4.89-5.01 (m, 3H), 5.69 (m, 1H), 6.12 (s, 1H), 6.47 (m, 1H), 6.54 (m, 1H), 6.70 (s, 1H), 6.72 (m, 2H), 6.87 (m, 1H), 6.97 (m, 2H), 7.10 (m, 2H), 7.18 (m, 1H), 7.25 (s, 1H), 7.29-7.30 (m, 2H), 7.59 (d, 1H, J = 9.0 Hz), 7.81 (d, 1H, J = 9.0 Hz), 8.38 (s, 1H), 8.47 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.3, 14.4, 28.4, 28.6, 28.9, 30.8, 41.5, 46.2, 49.5, 50.5, 51.8, 51.9, 52.8, 57.6, 61.5, 69.1, 69.2, 112.1, 112.5, 113.4, 114.3, 114.7, 115.0, 115.4, 122.1, 122.3, 122.8, 124.7, 125.5, 126.3, 127.3, 127.4, 128.7, 128.8, 129.1, 129.4, 129.9, 130.0, 131.4, 131.6, 133.7, 135.4, 135.5, 136.2, 157.4, 157.7, 157.9, 160.8, 160.9, 161.4, 163.2, 163.6, 164.8, 168.3.

Preparation of **ethyl 3-(1-(*N*-(4-(benzyloxy)benzyl)formamido)-2-(tert-butylamino)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (99b):** The product was purified by chromatography on silica gel (petroleum ether/ethyl acetate, 1:1) as yellow solid (162 mg, yield: 70%). SFC/MS: t_R = 3.89 min; m/z = 574.38 [M-H]$^-$. HRMS: C$_{32}$H$_{35}$ClN$_3$O$_5$, 576.2265 (calcd.), 576.2272 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.21 (s, 9H), 1.30 (s, 6H), 1.35-1.38 (m, 5H), 4.24 (ABd, 1H, J = 16.2 Hz), 4.30-4.34 (m, 4H), 4.39 (m, 1H), 4.51 (ABd, 1H, J = 16.2 Hz), 4.75 (ABd, 1H, J = 15.0 Hz), 4.97-5.05 (m, 3H), 5.61 (s, 1H), 5.66 (s, 1H), 6.13 (s, 1H), 6.45 (m, 1H), 6.56 (m, 1H), 6.70 (s, 1H), 6.77 (m, 1H), 6.93 (m, 1H), 6.99 (m, 1H), 7.10-7.20 (m, 4H), 7.33-7.37 (m, 2H), 7.39-7.43 (m, 8H), 7.59 (d, 1H, J = 8.4 Hz), 7.83 (d, 1H, J = 9.0 Hz), 8.42 (s, 1H), 8.49 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.3, 14.4, 28.4, 28.6, 28.9, 30.9, 41.6, 46.2, 49.6, 51.8, 51.9, 52.8, 57.6, 61.5, 61.6, 69.8, 69.9, 70.0, 112.0, 112.4, 113.5, 114.4, 114.8, 115.1, 115.6, 122.1, 122.3, 122.8, 124.8, 125.6, 126.2, 127.2, 127.3, 127.4, 127.5, 128.0, 128.6, 129.2, 129.4, 129.7, 129.9, 131.5, 131.6, 136.0, 136.1, 137.0, 137.1, 157.6, 158.0, 160.7, 160.8, 163.5, 164.8, 168.3.

Preparation of **ethyl 3-(2-(*tert*-butylamino)-1-(*N*-(4-(4-nitrobenzyloxy)benzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylate (99c):** The product was purified by chromatography on silica gel (petroleum ether/ethyl acetate, 1:1) as yellowish solid (55 mg, yield: 44%). SFC/MS: t_R = 4.37 min; m/z = 621.31 [M+H]$^+$. HRMS: $C_{32}H_{33}ClN_4O_7Na$, 643.1935 (calcd.), 643.1945 (found). ^{1}H NMR (600 MHz, CDCl$_3$, a mixture of rotamers): 1.23 (s, 9H), 1.31 (s, 6H), 1.37-1.43 (m, 6H), 4.28-4.31 (m, 2H), 4.36-4.40 (m, 3H), 4.57 (ABd, 1H, *J* = 15.6 Hz), 4.82 (ABd, 1H, *J* = 15.0 Hz), 5.06-5.12 (m, 3H), 5.48 (s, 1H), 5.54 (s, 1H), 6.12 (s, 1H), 6.52 (m, 1H), 6.57 (m, 1H), 6.70 (s, 1H), 6.75 (m, 1H), 6.99 (m, 2H), 7.14-7.16 (m, 2H), 7.32 (s, 1H), 7.56-7.64 (m, 6H), 7.87 (m, 1H), 8.05 (s, 1H), 8.25-8.31 (m, 4H), 8.42 (s, 1H), 8.50 (s, 1H), 9.24 (br.s, 1H), 9.50 (br.s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, a mixture of rotamers): 14.4, 28.4, 28.6, 28.9, 30.9, 46.1, 49.5, 51.9, 52.6, 57.5, 61.7, 68.6, 68.7, 111.8, 112.2, 113.6, 114.2, 114.6, 115.8, 122.4, 122.5, 123.0, 123.8, 124.9, 125.7, 126.1, 127.2, 127.4, 127.5, 127.6, 129.5, 130.5, 131.7, 131.9, 135.9, 136.0, 144.4, 144.5, 147.6, 157.1, 157.3, 160.6, 160.7, 163.0, 163.5, 164.6, 168.0, 168.1.

General procedure for the synthesis of compounds 100:

Preparation of **3-(2-(*tert*-butylamino)-1-(*N*-(4-(4-chlorobenzyloxy)benzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylic acid (100a):** The mixture of **99a** (53 mg), EtOH (0.5 mL), water (0.5 mL), LiOH (20 mg) was stirring under RT for 2 days. The reaction mixture was acidified with 1M HCl (pH ~ 6), and extracted with DCM (10 mL x 3). The combined organic layer was dried over sodium sulfate, and evaporated. 35 mg of white solid (75%) was obtained. SFC/MS: t_R = 7.36 min; m/z = 580.29 [M-H]$^-$. HRMS: $C_{30}H_{29}Cl_2N_3O_5Na$, 604.1382 (calcd.), 604.1439 (found). ^{1}H NMR (600 MHz, CDCl$_3$, major rotamer): 1.15 (s, 9H), 4.34-4.37 (m, 2H), 5.02 (s, 2H), 6.52 (s, 1H), 6.59 (s, 1H), 6.80-6.82 (m, 2H), 7.00-7.02 (m, 1H), 7.08-7.09

(m, 2H), 7.37-7.43 (m, 6H), 7.72 (m, 1H), 8.36 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, major rotamer): 27.4, 27.5, 46.8, 50.8, 56.7, 68.5, 111.5, 113.9, 114.1, 114.2, 120.2, 121.4, 125.1, 126.9, 128.1, 128.2, 128.7, 128.9, 129.1, 129.4, 133.1, 135.3, 136.3, 157.8, 165.3, 169.9.

Preparation of **3-(1-(*N*-(4-(benzyloxy)benzyl)formamido)-2-(*tert*-butylamino)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylic acid (100b):** 42 mg of yellowish solid (71%) was obtained. SFC/MS: t_R = 7.23 min; *m/z* = 546.33 [M-H]$^-$. HRMS: C$_{30}$H$_{31}$ClN$_3$O$_5$, 548.1952 (calcd.), 548.1955 (found). ^{1}H NMR (600 MHz, CDCl$_3$, major rotamer): 1.17 (s, 9H), 4.36-4.39 (m, 2H), 5.06 (s, 2H), 6.54 (s, 1H), 6.85-6.86 (m, 2H), 7.02-7.04 (m, 1H), 7.11-7.13 (m, 2H), 7.33 (m, 2H), 7.33-7.45 (m, 6H), 7.74 (m, 1H), 8.37 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, major rotamer): 28.8, 28.9, 48.2, 52.2, 58.2, 70.9, 111.6, 112.9, 115.6, 121.6, 122.8, 126.5, 128.4, 128.5, 128.8, 129.5, 130.1, 130.2, 130.8, 136.7, 138.9, 159.5, 166.7, 171.2.

Preparation of **3-(2-(*tert*-butylamino)-1-(*N*-(4-(4-nitrobenzyloxy)benzyl)formamido)-2-oxoethyl)-6-chloro-1*H*-indole-2-carboxylic acid (100c):** 42 mg of yellow solid (80%) was obtained. HRMS: C$_{30}$H$_{28}$ClN$_4$O$_7$, 591.1647 (calcd.), 591.1703 (found). ^{1}H NMR (600 MHz, CDCl$_3$, major rotamer): 1.13 (s, 9H), 3.37 (s, 2H), 4.35 (m, 1H), 5.02 (m, 1H), 6.41-6.68 (m, 6H), 7.00-7.01 (m, 4H), 7.34-7.37 (m, 2H), 7.58 (m, 1H), 7.70-7.71 (m, 2H), 8.15 (m, 1H), 8.20 (m, 1H), 8.36 (s, 1H). ^{13}C NMR (150 MHz, CDCl$_3$, major rotamer): 27.4, 50.8, 56.9, 68.1, 111.7, 114.0, 114.5, 114.6, 115.5, 120.4, 121.6, 123.1, 123.2, 125.1, 127.3, 127.5, 129.0, 129.6, 130.2, 135.4, 145.1, 147.4, 156.3, 157.3, 169.7.

Synthesis of compounds 103 and 104:

Preparation of **ethyl 6-chloro-3-(2-oxo-1-(*N*-(3,4,5-trifluorobenzyl)formamido)-2-(2,4,4-trimethylpentan-2-ylamino)ethyl)-1*H*-indole-2-carboxylate (103):** The mixture of ethyl 6-chloro-3-formyl-1*H*-indole-2-carboxylate (0.2 mmol, 50.2 mg), (3,4,5-trifluorophenyl)

methanamine (0.2 mmol, 24.4 µL), 2-isocyano-2,4,4-trimethylpentane (0.2 mmol, 28 mg), formic acid (0.2 mmol, 7.9 µL) in 0.5 mL of methanol was stirring under RT for 5 days. The product was purified by chromatography on silica gel (petroleum ether/ethyl acetate, 2:1) as yellowish solid (30 mg, yield: 26%). HPLC/MS: t_R = 12.18 min; m/z = 579.7 [M+H]$^+$. HRMS: $C_{29}H_{33}ClF_3N_3O_4Na$, 602.2009 (calcd.), 602.1990 (found). ^{1}H NMR (400 MHz, CDCl$_3$, a mixture of rotamers): 0.85-0.86 (m, 13H), 1.03 (s, 3H), 1.33 (m, 4H), 1.40-1.46 (m, 12H), 1.80 (m, 2H), 4.17 (ABd, 1H, J = 15.6 Hz), 4.37-4.45 (m, 3H), 4.58 (ABd, 1H, J = 16.8 Hz), 4.98 (ABd, 1H, J = 15.6 Hz), 5.46-5.51 (m, 2H), 6.13 (s, 1H), 6.18 (m, 1H), 6.44 (m, 1H), 6.73 (s, 1H), 7.18-7.22 (m, 2H), 7.38-7.39 (m, 2H), 7.72 (d, 1H, J = 8.8 Hz), 7.91 (d, 1H, J = 8.8 Hz), 8.40 (s, 1H), 8.47 (s, 1H), 9.49 (br.s, 1H), 9.64 (br.s, 1H). ^{13}C NMR (100 MHz, CDCl$_3$, a mixture of rotamers): 14.3, 28.1, 28.2, 28.8, 28.9, 29.3, 30.9, 31.2, 31.3, 31.4, 31.5, 31.6, 45.7, 52.3, 52.4, 52.6, 56.1, 56.2, 57.0, 61.8, 61.9, 109.5, 109.7, 111.0, 111.1, 111.2, 112.0, 112.3, 114.9, 122.3, 122.6, 122.8, 123.0, 124.6, 125.4, 126.3, 127.2, 132.1, 132.4, 135.9, 160.5, 163.3, 164.3, 167.4, 167.5.

Preparation of **6-chloro-3-(2-oxo-1-(*N*-(3,4,5-trifluorobenzyl)formamido)-2-(2,4,4-trimethyl pentan-2-ylamino)ethyl)-1*H*-indole-2-carboxylic acid (104):** The mixture of **103** (26 mg), EtOH (0.5 mL), water (0.5 mL), LiOH (20 mg) was stirring under RT for 2 days. The reaction mixture was acidified with 1M HCl (pH ~ 6), and extracted with DCM (10 mL x 3). The combined organic layer was dried over sodium sulfate, and evaporated. 22 mg of white solid (89%) was obtained. HRMS: $C_{27}H_{30}ClF_3N_3O_4$, 552.1877 (calcd.), 552.1917 (found). ^{1}H NMR (400 MHz, CD$_3$OD, a mixture of rotamers): 0.88 (s, 12H), 1.03 (s, 2H), 1.29-1.42 (m, 10H), 2.04-2.07 (m, 2H), 4.25-4.33 (m, 2H), 4.69 (ABd, 1H, J = 16.4 Hz), 5.06 (ABd, 1H, J = 15.6 Hz), 6.21 (m, 1H), 6.27 (s, 1H), 6.43-6.46 (m, 2H), 6.76 (s, 1H), 7.15-7.18 (m, 2H), 7.36-7.50 (m, 3H), 7.83-7.89 (m, 2H), 8.39 (s, 1H), 8.42 (s, 1H). ^{13}C NMR (100 MHz, CD$_3$OD, a mixture

of rotamers): 25.8, 27.0, 28.7, 28.8, 28.9, 29.3, 29.4, 44.1, 47.6, 49.3, 49.5, 51.1, 53.9, 55.5, 107.8, 108.0, 109.1, 109.2, 109.3, 110.2, 110.4, 110.9, 112.1, 119.7, 119.8, 120.3, 120.5, 123.2, 124.0, 126.5, 129.0, 129.2, 133.0, 134.7, 160.8, 163.0, 163.6, 167.8.

4.2.2 Directions for long-term study

The receptor-based drug discovery approach of p53-Mdm2 inhibitors has been firmly established, which allows to generate diverse lead compounds for further optimization. A worthwhile goal now within reach is the identification of potent p53-Mdm4 specific inhibitors, and p53-Mdm2/Mdm4 dual functional inhibitors. In the future, it also can be expected that this approach would be useful to generate small molecules to target other PPIs taking advanatage of available 3D structural information.

The ligand-based drug discovery approach facilitates the generation of drug-like compound to cover unexplored chemical space, which would be specific to target PPIs rather than traditional drug targets. Based on the advantages of MCRs, piliot libraries of anchor-biased compounds would be achieved via diversity-oritented synthesis, and available to the community for the screening of small molecule probes targeting PPIs.

In a view of the future, the efforts of structure-based drug discovery would provide more and more successful examples of seeking small molecule inhibitors of specific PPIs. The application and/or integration of receptor- and ligand-based drug discovery approaches would certainly accelerate the identification of drug candidates targeting PPIs.

APPENDIX A. GENERAL METHODS

Chemistry

All reagents were purchased from commercial sources and used without further purification. The reactions were conducted under air atmosphere unless otherwise indicated. Analytical thin-layer chromatography (TLC) was performed on SiO_2 plates 250 µm on Alumina from Whatman. Visualization was accomplished by UV irradiation at 254 nm. Proton and carbon NMR spectra were determined on Bruker 400 or 600 MHz NMR spectrometers. Chemical shifts are reported as δ values in parts per million (ppm) as referenced to residual solvent. ^{1}H NMR spectra are tabulated as follows: chemical shift, number of protons, multiplicity (s = singlet, br.s = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet), and coupling constant. High resolution mass spectra were obtained at the University of Pittsburgh Mass Spectrometry facility. LC-MS analysis was performed on an Shimadzu instrument, using an analytical C18 column (Dionex Acclaim 120 Å, 2.1 × 50 mm, 3.0 µm, 0.2 mL/min) coupled to an Applied Biosystems API2000 mass spectrometer. Acetonitrile/water mixtures were used as the mobile phase for reverse-phase HPLC coupled to electrospray ionization-mass spectrometry (ESI-MS).

NMR spectra of some compounds showed clearly the presence of two rotamers under room temperature, which are not chromatographically separable. The population of rotamers was found related to the deuterium solvent used, e.g. compound **79d** (**Appendix Figure 1**).
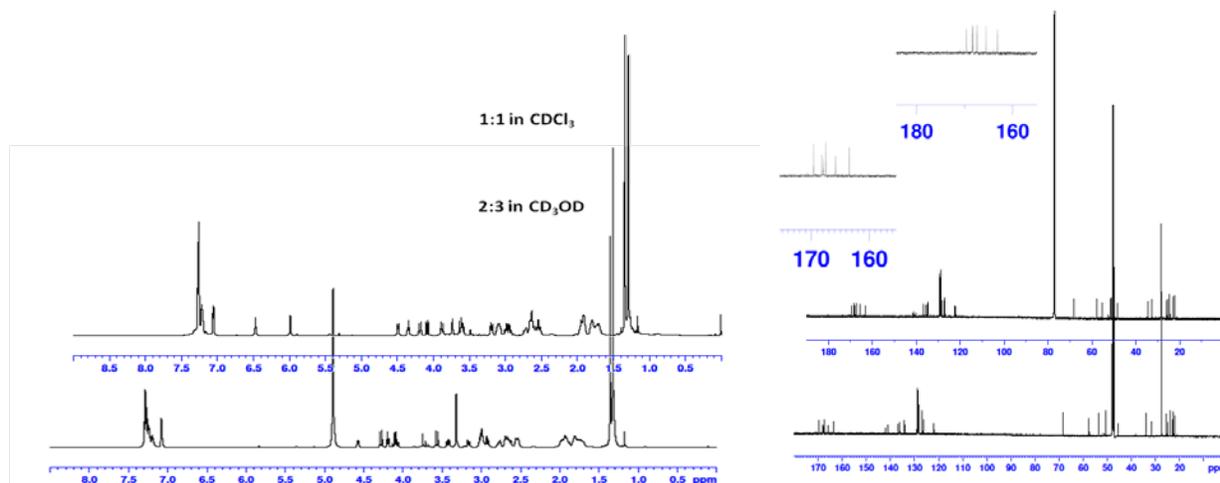
Appendix Figure 1. NMR spectra of compound **79d**

FP and NMR binding assays (in collaboration with Dr. Holak's laboratory)

Human recombinant Mdm2 (residues 1-118) and p53 (residues 1-321) were expressed and purified as described in Bista et al., 2009.[118] Uniform ^{15}N labeling was achieved by growing *Escherichia coli* BL21(DE3) RIL in M9 minimal medium containing ^{15}NH$_4$Cl as nitrogen source.[340] The SEI-AIDA experiment was performed according to Bista et al., 2009.[118] 14 μM Mdm2/p53 complex was mixed with compounds in 1:1 molar ratio; amount of p53 released from the complex by the compound was estimated from 1D spectrum and K$_d$ of Mdm2-inhibitor interaction was calculated according to Krajewski et al., 2007.[117] FP binding assays were performed according to Czarna et al., 2009.[124] Binding constant and inhibition curves were fitted using the SigmaPlot (SPSS Science Software).

^{1}H-^{15}N HSQC spectra were acquired using the fast-HSQC pulse sequence (Mori et al., 1995).[341] For the ^{1}H-^{15}N HSQC spectrum, a total of 2048 complex points in t2 and 192 t1 increments were acquired. NMR data were processed using the Bruker program Xwin-NMR version 3.5. Titration experiments were performed using a series of ^{1}H-^{15}N HSQC of labeled Mdm2 or unlabeled Mdm2 along with the unlabelled partner. 10% D$_2$O was added to NMR

samples to provide lock signal. 100mM stock solutions of the compounds were prepared by dissolving them in perdeuterated DMSO. For HSQC titration, 0.2 mM ^{15}N-labeled Mdm2 was mixed with compounds in 1:1 and 1:5 protein: compound molar ratio. Normalized chemical shift perturbations were calculated according to equation (Stoll et al., 2001):[33]

$$\Delta\delta = \sqrt{0.04\left(\delta_N^{bound} - \delta_N^{free}\right)^2 + \left(\delta_H^{bound} - \delta_H^{free}\right)^2}$$

APPENDIX B. CHIRAL SEPARATION

Chiral separation of 8l:

The enantiomers of **8l** in 20 mg amounts were conveniently obtained by chiral separation via Supercritical Fluid Chromatography (SFC). Experiments were performed using the analytical SFC-MS Resolution System (Waters 2998 Photodiode Array Detector, Waters 3100 Mass Detector), and the preparative TharSFC System (Waters 2998 Photodiode Array Detector). Both analytical and preparative SFC columns were operating at 40 $^{\circ}$C in Analytical-2-Prep Column Oven. Carbon dioxide supplied by BDS 500 Gas Delivery System was used as the primary mobile phase for SFC.
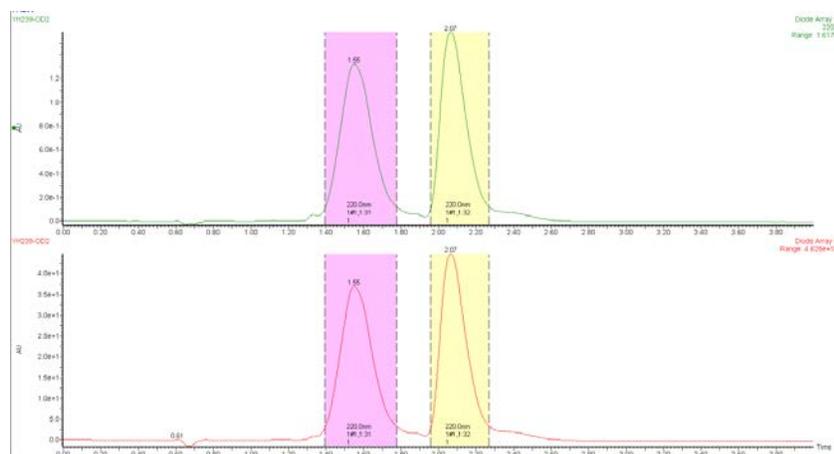
A. Analytical SFC (upper panel: UV, lower panel: MS). Column: RegisCell (#784104, OD), 5 µM particle size, 250 mm x 4.6 mm i.d.
Flow rate: 4 mL/min
Modifier: isocratic elution with 20% ethanol
Injection volume: 5 µL

Appendix Figure 2. Chiral separation of lead compound **8l**

Appendix Table 1. Optical rotations

The enantiomers of **8l** were separated by preparative SFC: enantiomer 1 (t_R = 1.55 min), enantiomer 2 (t_R = 2.07 min). The enantiomers of **9l** were obtained by hydrolysis of the corresponding

B. Preparative SFC (upper panel: UV 220nm, lower panel: UV).
Column: RegisCell (#784106, OD), 5 µM particle size, 250 mm x 21.1 mm i.d.
Flow rate: 100 g/min
Modifier: isocratic elution with 20% ethanol
Injection volume: 500 µL

	enantiomer 1	enantiomer 2
8l	$[\alpha]_D$ = +144.0° (c = 0.4) K_i = 2 µM (Mdm2)	$[\alpha]_D$ = -140.6° (c = 0.3) Ki = 5 µM (Mdm2)
9l	$[\alpha]_D$ = +50.6° (c = 1.1) Ki = 0.3 µM (Mdm2)	$[\alpha]_D$ = -51.1° (c = 0.3) Ki = 0.7 µM (Mdm2)

enantiomers of **8l** (Method D). The optical rotations and binding affinities with Mdm2 (FP assay) are shown in **Table 1**. Optical rotations were measured using Perkin Elmer 241 Polarimeter at 20 °C in a 10 cm cell in methanol.

Chiral separation of 17e:

The enantiomers of **17e** in 20 mg amounts were conveniently obtained by chiral separation via Supercritical Fluid Chromatography (SFC). Experiments were performed using the analytical SFC-MS Resolution System (Waters 2998 Photodiode Array Detector, Waters 3100 Mass Detector), and the preparative TharSFC System (Waters 2998 Photodiode Array

Detector). Both analytical and preparative SFC columns were operating at 40 ^{o}C in Analytical-2-Prep Column Oven. Carbon dioxide supplied by BDS 500 Gas Delivery System was used as the primary mobile phase for SFC.

A. Analytical SFC (upper panel: UV, lower panel: MS). Column: RegisCell (#784104, OD), 5 µM particle size, 250 mm x 4.6 mm i.d.
Flow rate: 4 mL/min
Modifier: isocratic elution with 20% ethanol
Injection volume: 5 µL

B. Preparative SFC (upper panel: UV 220nm, lower panel: UV). Column: RegisCell (#784106, OD), 5 µM particle size, 250 mm x 21.1 mm i.d.
Flow rate: 100 g/min
Modifier: isocratic elution with 20% ethanol
Injection volume: 500 µL

Appendix Figure 3. Efficient enantiomer seperation of lead compound **17e** using preparative SFC

The enantiomers of **17e** were separated by preparative SFC: enantiomer 1 (t_R = 1.28 min), enantiomer 2 (t_R = 1.66 min). The enantiomers of **18e** were obtained by hydrolysis of the corresponding enantiomers of **17e**. The optical rotations and binding affinities with Mdm2 (FP assay) are shown in **Table 2**. Optical rotations were measured using Perkin Elmer 241 Polarimeter at 20 ^{o}C in a 10 cm cell in methanol.

Appendix Table 2. Optical rotation of the enantiomers of **17e** and **18e**

	enantiomer 1	enantiomer 2
17e	$[\alpha]_D$ = +119.6° (c = 0.8) K_i = 0.9 µM (Mdm2)	$[\alpha]_D$ = -114.6° (c = 0.7) Ki = 30.5 µM(Mdm2)
18e	$[\alpha]_D$ = +54.4° (c = 0.6) Ki = 0.2 µM (Mdm2)	$[\alpha]_D$ = -50.7° (c = 0.4) Ki = 0.4 µM (Mdm2)

Chiral separation of 17m:

The enantiomers of **17m** in 20 mg amounts were conveniently obtained by chiral separation via Supercritical Fluid Chromatography (SFC). Experiments were performed using the analytical SFC-MS Resolution System (Waters 2998 Photodiode Array Detector, Waters 3100 Mass Detector), and the preparative TharSFC System (Waters 2998 Photodiode Array Detector). Both analytical and preparative SFC columns were operating at 40 ^{o}C in Analytical-2-Prep Column Oven. Carbon dioxide supplied by BDS 500 Gas Delivery System was used as the primary mobile phase for SFC.

A. Analytical SFC (upper panel: UV, lower panel: MS). Column: RegisCell (#784104, OD), 5 µM particle size, 250 mm x 4.6 mm i.d.
Flow rate: 4 mL/min
Modifier: isocratic elution with 20% ethanol
Injection volume: 5 µL

B. Preparative SFC (upper panel: UV 220nm, lower panel: UV).
Column: RegisCell (#784106, OD), 5 µM particle size, 250 mm x 21.1 mm i.d.
Flow rate: 100 g/min
Modifier: isocratic elution with 20% ethanol
Injection volume: 500 µL

Appendix Figure 4. Efficient enantiomer seperation of lead compound **17m** using preparative SFC

Appendix Table 3. Optical rotation of the enantiomers of **17m** and **18m**

The enantiomers of **17m** were separated by preparative SFC: enantiomer 1 (t_R = 1.22 min), enantiomer 2 (t_R = 1.63 min). The enantiomers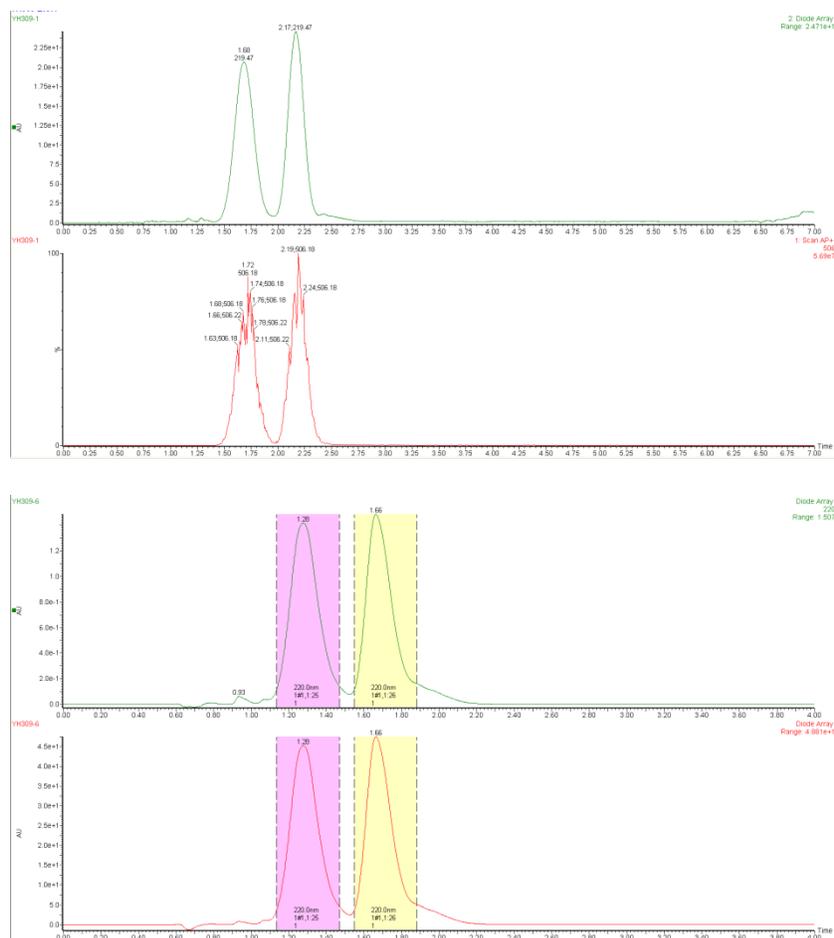 of **18m** were obtained by hydrolysis of the corresponding enantiomers of **17m**. The optical rotations and binding affinities with Mdm2 (FP assay) are shown in **Table 3**.

	enantiomer 1	enantiomer 2
17m	$[\alpha]_D$ = +107.6° (c = 0.3) K_i = 0.4 µM (Mdm2)	$[\alpha]_D$ = -104.8° (c = 0.3) Ki = 16.7 µM(Mdm2)
18m	$[\alpha]_D$ = +37.7° (c = 0.3) Ki = 0.1 µM (Mdm2)	$[\alpha]_D$ = -35.4° (c = 0.3) Ki = 0.28 µM (Mdm2)

Optical rotations were measured using Perkin Elmer 241 Polarimeter at 20 °C in a 10 cm cell in methanol.

APPENDIX C. AQUEOUS SOLUBILITY

Aqueous solubility of 9l:

Standard curve of **9l**

      0.66 mg of **9l** was dissolved in 15 mL of 10 mM pH=7.0 Na_3PO_4 buffer (concentration = 44 µg/mL). The solution was sonicated in water bath for 30 min. The stock solution was then diluted to 35.2 µg/mL, 26.4 µg/mL, 17.6 µg/mL, 8.8 µg/mL with 10 mM Na_3PO_4 buffer (pH=7.0). The UV absorbance (200 µL of each solution) was measured using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA) in UV-transparent 96 well plates.
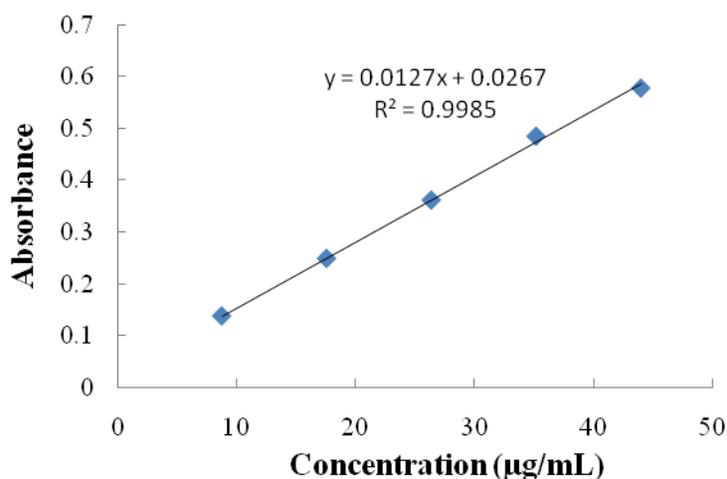
Appendix Figure 5. The standard curve of **9l** (λ=302 nm)

The preparation of saturated solution of **9l** and the UV measurement

150 µL of Na_3PO_4 buffer (pH = 7.0) was added to dissolve 0.97 mg of **9l**. The sample was sonicated in water bath for 30 min, and then shaked with the Bioshaker (BIONEXUS, Inc., Oakland, CA) at 1000 rpm and 20 ^oC for 12 h. The mixture was then centrifuged at 3000 rpm for 10 min. The supernatant was diluted with Na_3PO_4 buffer in 50 times for the UV quantification. The absorbance at λ=302 nm was measured to be 0.36, which is corresponding to 26.24 µg/mL based on the standard curve. Thus, the solubility of **9l** in 10 mM pH = 7.0 Na_3PO_4 buffer at 20 ^oC is 1.31 mg/mL.

Aqueous solubility of 18m:

Standard curve of **18m**

0.65 mg of **18m** was dissolved in 15 mL of 10 mM pH=7.0 Na_3PO_4 buffer (concentration = 43.3 ug/mL). The solution was sonicated in water bath for 30 min. The stock solution was then diluted to 34.7 µg/mL, 26.0 µg/mL, 17.3 µg/mL, 8.67 µg/mL with 10 mM Na_3PO_4 buffer (pH=7.0). The UV absorbance (200 µL of each solution) was measured using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA) in UV-transparent 96 well plates.

Standard Curve of 7m

$y = 0.0118x + 0.0252$
$R^2 = 0.9992$
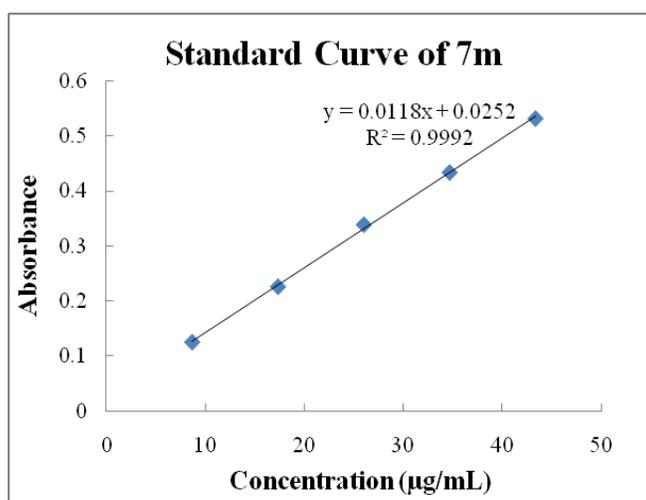
Appendix Figure 6. Solubility determination of **18m**. The standard curve of **18m** (λ=302 nm)

The preparation of saturated solution of **18m** and the UV measurement

150 µL of Na$_3$PO$_4$ buffer (pH = 7.0) was added to dissolve 1.05 mg of **18m**. The sample was sonicated in water bath for 30 min, and then shaked with the Bioshaker (BIONEXUS, Inc., Oakland, CA) at 1000 rpm and 20 ^{o}C for 12 h. The mixture was then centrifuged at 3000 rpm for 10 min. The supernatant was diluted with Na$_3$PO$_4$ buffer in 50 times for the UV quantification. The absorbance at λ=302 nm was measured to be 0.226, which is corresponding to 17.02 µg/mL based on the standard curve. Thus, the solubility of **18m** in 10 mM pH = 7.0 Na$_3$PO$_4$ buffer at 20 ^{o}C is **0.85 mg/mL**.

*Note: nutlin-3a has a solubility of **0.1 mg/mL** in a 1:10 solution of ethanol:PBS (refer to: Cayman Chemical Item Number 18585, http://www.caymanchem.com)

Aqueous solubility of 22b (YH245):

Standard curve of YH245

1.09 mg of YH245 was dissolved in 10 mL of 10 mM pH=7.0 Na$_3$PO$_4$ buffer (concentration = 0.109 mg/mL). The solution was sonicated in water bath for 30 min. The stock solution was then diluted to 10.9 µg/mL (5/10), 21.8 µg/mL (4/10), 32.7 µg/mL (3/10), 43.6 µg/mL (2/10) and 54.5 µg/mL (1/10) by 10 mM Na$_3$PO$_4$ buffer (pH=7.0). The UV absorbance was measured using a Hewlett-Packard 8452A UV-Vis diode array spectrophotometer (Palo Alto, CA).

Appendix Figure 7. The UV absorbance of YH245 in Na_3PO_4 buffer

$y = 18.39156x - 0.05015, R^2 = 0.99802$
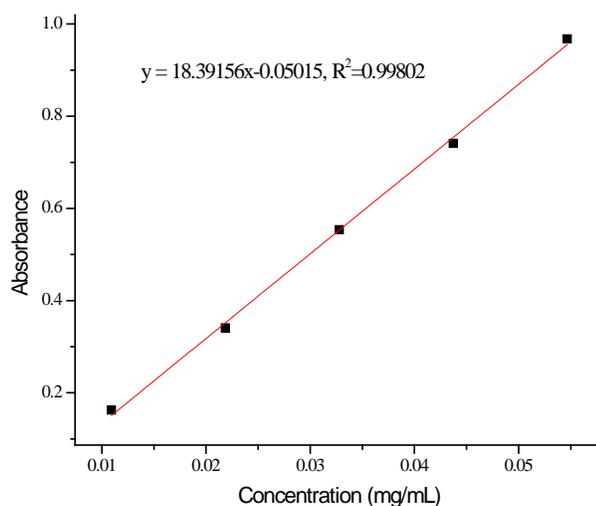
Appendix Figure 8. The standard curve of YH245 (λ=301 nm)

The preparation of saturated solution of YH 245 and the UV measurement

1 mL of Na_3PO_4 buffer (pH = 7.0) was added to dissolve 3.39 mg of YH245. The sample was sonicated in water bath for 30 min, and then shaked with the Bioshaker (BIONEXUS, Inc., Oakland, CA) at 1000 rpm and 20 ^{o}C for 12 h. The mixture was then

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centrifuged at 3000 rpm for 10 min. 100 μL of the supernatant was diluted with Na_3PO_4 buffer (50 times) for the UV quantification. The absorbance at $\lambda=301$ nm was measured to be 0.431, which is corresponding to 26.1 µg/mL based on the standard curve. Thus, the solubility of YH245 in 10 mM pH = 7.0 Na_3PO_4 buffer at 20 ^{o}C is 1.31 mg/mL.

Aqueous solubility of 21c (YH263):

Standard curve of YH263

0.81 mg of YH263 was dissolved in 40 mL of 10 mM pH=7.0 Na_3PO_4 buffer (concentration = 20.25 ug/mL). The solution was sonicated in water bath for 30 min. The stock solution was then diluted to 16.88 µg/mL, 13.50 µg/mL, 10.13 µg/mL, 6.75 µg/mL, 3.38 µg/mL by 10 mM Na_3PO_4 buffer (pH=7.0). The fluorescence was measured using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA) in UV-transparent 96 well plates (Ex: 250 nm; Em: 415 nm).

YH263 standard curve

$y = 5.0869x - 0.6633$
$R^2 = 0.9993$
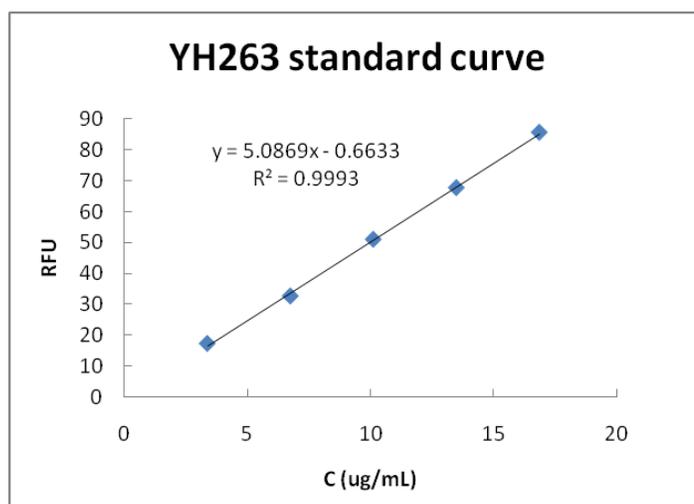
Appendix Figure 9. The standard curve of YH263 (Ex: 250 nm; Em: 415 nm)

The preparation of saturated solution of YH 263 and the fluorescence measurement

200 µL of Na₃PO₄ buffer (pH = 7.0) was added to dissolve 1.13 mg of YH263. The sample was sonicated in water bath for 30 min, and then shaked with the Bioshaker (BIONEXUS, Inc., Oakland, CA) at 1000 rpm and 20 $^\circ$C for 12 h. The mixture was then centrifuged at 3000 rpm for 10 min. The supernatant was diluted with Na₃PO₄ buffer in 10 times for the fluorescence quantification. The fluorescence at λ=415 nm (Ex: 250 nm) was measured to be 18.60, which is corresponding to 3.79 µg/mL based on the standard curve. Thus, the solubility of YH263 in 10 mM pH = 7.0 Na₃PO₄ buffer at 20 $^\circ$C is 37.9 µg/mL.

Aqueous solubility of 22c (YH264):

Standard curve of YH264

0.51 mg of YH264 was dissolved in 10 mL of 10 mM pH=7.0 Na₃PO₄ buffer (concentration = 51 ug/mL). The solution was sonicated in water bath for 30 min. The stock solution was then diluted to 38.25 µg/mL, 25.5 µg/mL, 12.75 µg/mL, 6.375 µg/mL 10 mM Na₃PO₄ buffer (pH=7.0). The UV absorbance was measured using a SpectraMax M2 microplte reader (Molecular Devices, Sunnyvale, CA) in UV-transparent 96 well plates.
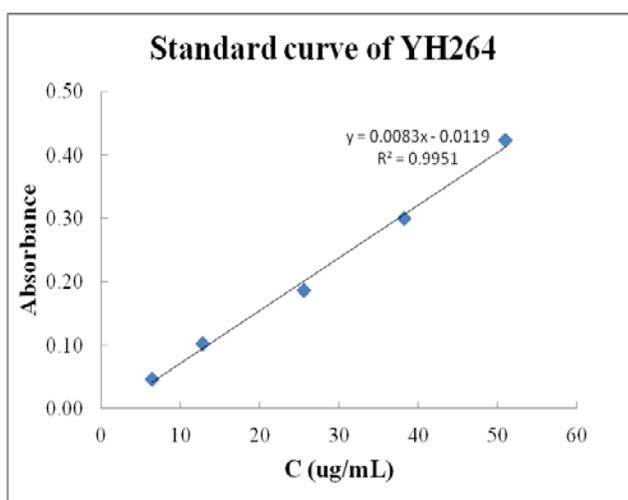
Appendix Figure 10. The standard curve of YH264 (λ=300 nm)

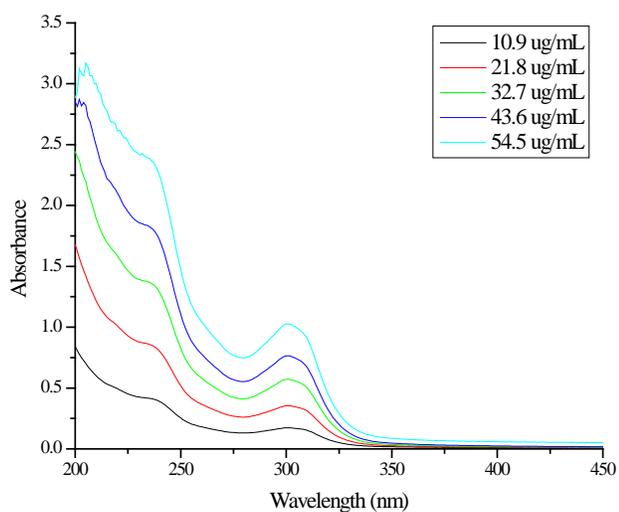The preparation of saturated solution of YH 264 and the UV measurement

150 µL of Na$_3$PO$_4$ buffer (pH = 7.0) was added to dissolve 0.835 mg of YH264. The sample was sonicated in water bath for 30 min, and then shaked with the Bioshaker (BIONEXUS, Inc., Oakland, CA) at 1000 rpm and 20 ^{o}C for 12 h. The mixture was then centrifuged at 3000 rpm for 10 min. The supernatant was diluted with Na$_3$PO$_4$ buffer in 60 times for the UV quantification. The absorbance at λ=301 nm was measured to be 0.218, which is corresponding to 27.72 µg/mL based on the standard curve. Thus, the solubility of YH264 in 10 mM pH = 7.0 Na$_3$PO$_4$ buffer at 20 ^{o}C is 1.66 mg/mL.

Aqueous solubility of 24c (YH265):

Standard curve of YH265

0.72 mg of YH265 was dissolved in 10 mL of 10 mM pH=7.0 Na$_3$PO$_4$ buffer (concentration = 72 ug/mL). The solution was sonicated in water bath for 30 min. The stock solution was then diluted to 63.6 µg/mL, 42 µg/mL, 20.4 µg/mL, 8.4 µg/mL with 10 mM Na$_3$PO$_4$ buffer (pH=7.0). The UV absorbance was measured using a SpectraMax M2 microplte reader (Molecular Devices, Sunnyvale, CA) in UV-transparent 96 well plates.
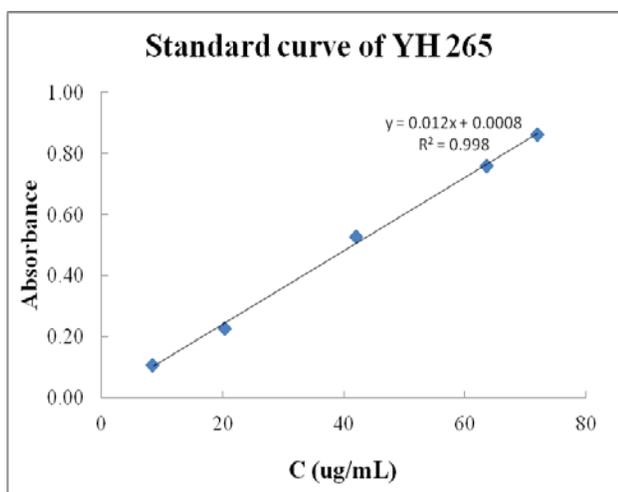
Appendix Figure 11. The standard curve of YH265 (λ=300 nm)

The preparation of saturated solution of YH 265 and the UV measurement

150 µL of Na$_3$PO$_4$ buffer (pH = 7.0) was added to dissolve 1.295 mg of YH265. The sample was sonicated in water bath for 30 min, and then shaked with the Bioshaker (BIONEXUS, Inc., Oakland, CA) at 1000 rpm and 20 ^{o}C for 12 h. The mixture was then centrifuged at 3000 rpm for 10 min. The supernatant was diluted with Na$_3$PO$_4$ buffer in 12 times for the UV quantification. The absorbance at λ=301 nm was measured to be 0.578, which is corresponding to 45.02 µg/mL based on the standard curve. Thus, the solubility of YH265 in 10 mM pH = 7.0 Na$_3$PO$_4$ buffer at 20 ^{o}C is 0.54 mg/mL.

APPENDIX D. NCI-60 SCREENING

| Developmental Therapeutics Program One Dose Mean Graph | NSC: D-754939 / 1 | Conc: 1.00E-5 Molar | Test Date: Dec 06, 2010 |
| | Experiment ID: 1012OS69 | | Report Date: Jan 08, 2011 |

Panel/Cell Line	Growth Percent
Leukemia	
CCRF-CEM	-17.30
RPMI-8226	-40.84
Non-Small Cell Lung Cancer	
A549/ATCC	37.52
EKVX	35.42
HOP-62	-31.87
HOP-92	-10.12
NCI-H226	52.91
NCI-H23	42.86
NCI-H322M	32.96
NCI-H460	-82.89
NCI-H522	-69.63
Colon Cancer	
COLO 205	-76.46
HCC-2998	16.77
HCT-116	-97.38
HCT-15	-46.42
KM12	-87.91
SW-620	-87.90
CNS Cancer	
SF-268	10.50
SF-295	-50.40
SF-539	-46.21
SNB-19	2.70
SNB-75	36.31
U251	-55.87
Melanoma	
LOX IMVI	-55.81
MALME-3M	-64.25
M14	-79.77
MDA-MB-435	-64.59
SK-MEL-2	-29.63
SK-MEL-28	-89.95
SK-MEL-5	-37.93
UACC-257	-69.28
UACC-62	-63.87
Ovarian Cancer	
IGROV1	4.46
OVCAR-3	-42.23
OVCAR-4	65.79
OVCAR-5	65.61
OVCAR-8	-65.57
NCI/ADR-RES	54.60
SK-OV-3	87.70
Renal Cancer	
786-0	44.10
A498	56.59
ACHN	51.36
CAKI-1	37.81
RXF 393	13.72
SN12C	31.01
TK-10	-6.93
UO-31	21.57
Prostate Cancer	
PC-3	30.43
DU-145	2.82
Breast Cancer	
MCF7	-80.28
MDA-MB-231/ATCC	-51.28
HS 578T	35.28
BT-549	70.73
T-47D	54.65
MDA-MB-468	-85.65
Mean	-12.58
Delta	84.80
Range	185.08

Appendix Figure 12. Growth inhibition screening of **24c** (YH265)

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Appendix Figure 13. GI$_{50}$ determination of **24c** (YH265)

Appendix Figure 14. GI$_{50}$ determination of **21c** (YH263)

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APPENDIX E. VIRTUAL LIBRARIES

Appendix Table 4. MCRs used in the generation of anchor-biased library. These reactions, together with a set of roughly 1000 commercially available starting materials, define a theoretical chemical space of more than three trillion distinct chemical compounds. For example, requiring at least one indole starting material in each reaction yields as many as 190 billion compounds containing a tryptophan mimic. We created our prototype tryptophan-biased virtual library by randomly drawing indole-containing compounds from a diverse set of 20 multi-component reactions. A total of 54,000 reactions were performed using randomly chosen reactants and ChemAxons REACTOR software (http://www.chemaxon.com). OpenEye OMEGA (http://eyesopen.com/) was used with the default settings to enumerate 591,227 stereoisomers and generate 97.9 million conformations.

Reaction	Scheme
1	R^1CO_2H + R^2NH_2 + R^3CHO + R^4NC ⟶ product
2	R^1NHR^2 + $R^3C(O)R^4$ + R^5NC ⟶ product
3	R^1NHR^2 + $R^3C(O)R^4$ + R^5NC + HN_3 ⟶ tetrazole product
4	R^1NH_2 + $R^2C(O)R^3$ + R^4NC + HNCO ⟶ imidazolone product
5	amino acid (HO_2C–CHR^1–NH_2) + R^2CHO + R^3NC + MeOH ⟶ MeO_2C product
6	H_2N–CHR^1–CH_2CO_2H + R^2CHO + R^3NC ⟶ β-lactam product

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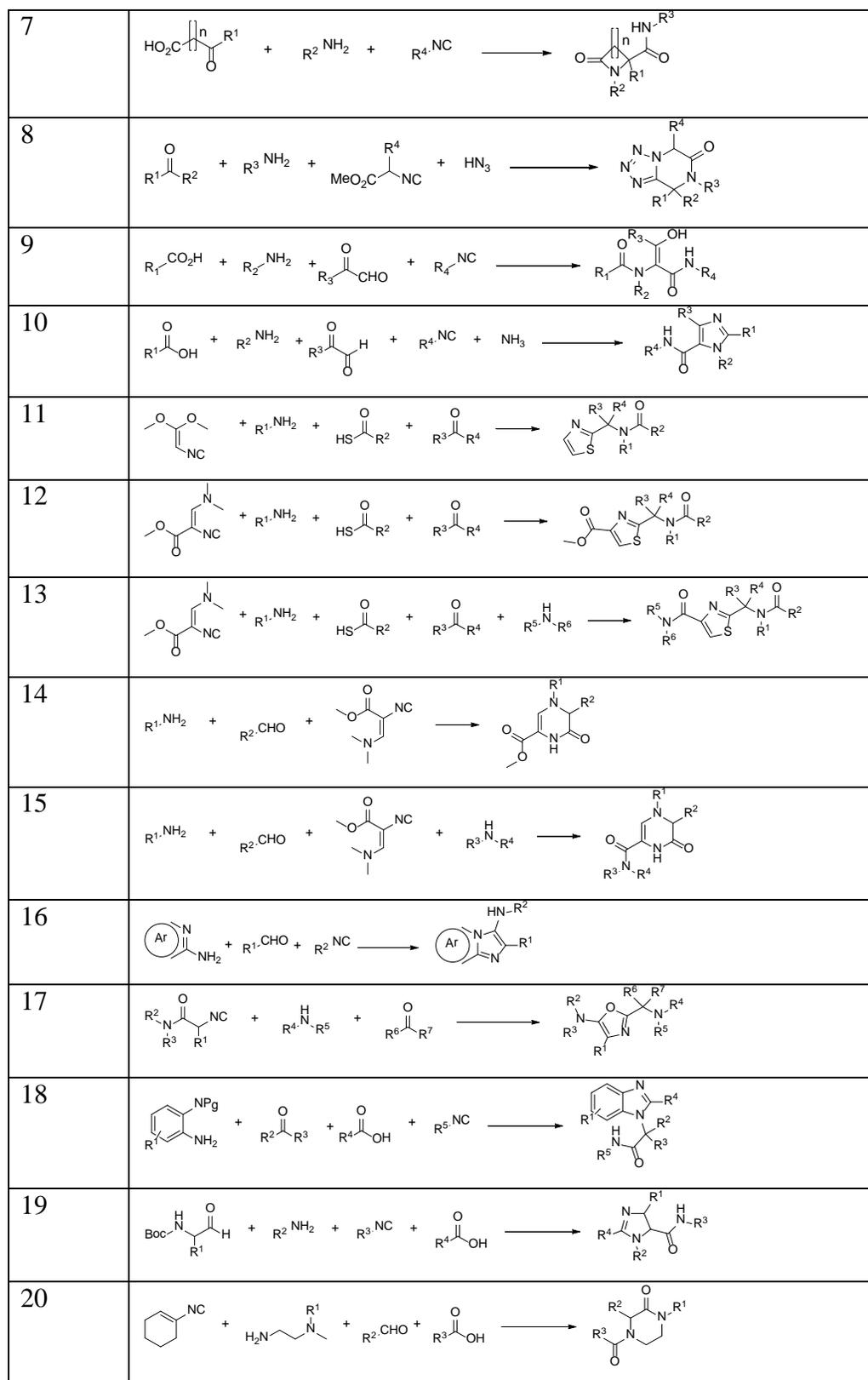
APPENDIX F. LIST OF PUBLICATIONS

1. <u>Huang Y</u>,[†] Wolf S,[†] Koes D, Popowicz GM, Camacho CJ, Holak TA, Doemling A. *Exhaustive Fluorine Scanning towards potent p53-Mdm2 Antagonists*. **ChemMedChem**, *in press*. ([†]equal contribution).

2. Wolf S,[†] **<u>Huang Y</u>**,[†] Popowicz GM, Goda S, Holak TA, Doemling A. *Ugi Multicomponent Reaction Derived p53-Mdm2 Antagonists*. *submitted*. ([†]equal contribution).

3. Koes D, Khoury K, **<u>Huang Y</u>**, Wang W, Bista M, Popowicz GM, Wolf S, Holak T, Doemling A, Camacho CJ. *Large-scale Design, Synthesis and Validation of Small Molecule Protein-protein Antagonists*. *submitted*.

4. <u>Huang Y</u>, Doemling A. *The Gewald Multicomponent Reaction*. **Molecular Diversity** *2011*, 15, 3-33. (Review).

5. <u>Huang Y</u>, Wolf S, Bista M, Meireles L, Camacho C, Holak TA, Doemling A. *New 1,4-Thienodiazepine- 2,5-diones via MCR (I): Synthesis, Virtual Space and p53-Mdm2 Activity*. **Chemcal Biology & Drug Design** *2010*, 76, 116-129.

6. <u>Huang Y</u>, Doemling A. *New 1,4-Thienodiazepine-2,5-diones via MCR (II): Scaffold Hopping from Gewald and Ugi-Deprotection-Cyclization (UDC) Approach*. **Chemcal Biology & Drug Design** *2010*, 76, 130-141.

7. Czarna A, Beck B, Srivastava S, Popowicz GM, Wolf S, **<u>Huang Y</u>**, Bista M, Holak TA, Doemling A. *Robust Generation of Lead Compounds for Protein-Protein Interactions by*

Computational and MCR Chemistry: p53-Hdm2 Antagonists. **Angewandte Chemie International Edition** *2010*, 49, 5352-5356.

8. Doemling A, **Huang Y**. *Piperazine Scaffolds via Isocyanide-based Multicomponent Reactions*. **Synthesis** *2010*, 2859-2883. (Review)

9. Wang K, Nguyen K, **Huang Y**, Doemling A. *Cyanoacetamide Multicomponent Reaction (I): Parallel Synthesis of Cyanoacetamides*. **Journal of Combinatorial Chemistry** *2009*, 11, 920-927.

10. Huang Y, Doemling A. *Multicomponent Reactions*. **Green Techniques for Organic Synthesis and Medicinal Chemistry**. Wiley, in press. (Book chapter)

11. Huang Y, Doemling A. *Isocyanide-based Multicomponent Reactions Towards Benzodiazepines*. **Isocyanide Chemistry: Applications in Synthesis and Material Science**. Wiley, in press. (Book chapter)

12. Huang Y, Khoury, K.; Doemling A. *The Piperazine Space in Isocyanide-based MCR Chemistry*. **Topics in Heterocyclic Chemistry**, Vol. 23: Synthesis of Heterocycles via Multicomponent Reactions. Orru R, Ruijter E (Eds.); Springer: New York, 2010; pp 85-127. (Book chapter)

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