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(54) Title: CYCLIC DESIGNER SCAFFOLDS FOR THE COVALENT TARGETING OF PROTEINS VIA MICHAEL ADDITION

(57) Abstract: Many biologically active natural products contain electrophilic Michael acceptor fragments. For example, curcumin and 4-hydroxyderricin contain an acyclic α,β-unsaturated ketone that alkylates cysteines. Other antitumor or anti-inflammatory herbal compounds such as Withaferin A or zerumbone contain cyclic α,β-unsaturated ketones and react with nucleophilic residues of proteins. These observations contributed to a paradigm shift in drug design and development in the last two decades: various drugs with covalent warhead have been developed and approved. Despite the apparent importance and success of covalent warheads in current drug design and developments, the applied warheads display a rather limited structural variance and complexity which automatically limits the attainable chemical space. Furthermore, to minimize possible side-reactions during the synthesis of drugs, the applied warheads are added appendages in the late-stage of the synthetic route, thus a warhead scaffold that can be synthetically easily varied using orthogonal chemistry and used as a tunable covalent warhead is still missing. Such a structurally more complex scaffold would be much more like the warheads of the natural products and is expected to be more selective in targeting nucleophiles found on the proteins. Moreover, owing to a larger contact surface, it might be more suitable for targeting shallow protein surfaces involved in protein-protein interactions.

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Cyclic designer scaffolds for the covalent targeting of proteins via Michael addition

Description

Background of the invention

Many biologically active natural products contain electrophilic Michael acceptor fragments (Gersch et al, 2012). For example, curcumin (from turmeric, *Curcuma longa*) and 4-hydroxyderricin (from *Angelica keiskei*) contain an acyclic (or open-chain) α,β-unsaturated ketone that alkylates cysteines. Other antitumor or anti-inflammatory herbal compounds such as Withaferin A (from winter cherry, *Withania somnifera*) or zerumbone (from ginger, *Zingiber zerumbet*) contain cyclic α,β-unsaturated ketones and react with nucleophilic residues (e.g., cysteines) of proteins (vimentin, NFκB or Keap1, HuR, respectively). These observations contributed to a paradigm shift in drug design and development in the last two decades: various drugs with a covalent warhead have been developed and approved.

Along this line, various new covalent drugs have been developed to block protein function via forming a covalent bond to a residue on the target protein (Sutanto et al, 2020) (Boike et al, 2022). Most often it is the cysteine, owing to its enhanced nucleophilicity, which is covalently modified by various electrophiles through Michael addition (Gehringer and Laufer, 2019). Following a modular approach, a cysteine targeting covalent drug is composed of a covalent warhead and a directing moiety: the former is used as a simple nonselective chemical warhead "appendage" (e.g., acrylamide), while the latter is a structurally more complex moiety that forms specific noncovalent contacts with the target protein. Accordingly, a covalent warhead is a modular, often transferable fragment of a more complex compound, where the former contributes to protein binding affinity by forming a covalent bond with the protein (reversible or irreversible). Its contribution to binding energy is distinct from those provided by noncovalent contacts between the protein and the small molecule. This design approach were successful in many cases which is reflected by the evolution of several approved and experimental drugs that bear α,β-unsaturated carbonyls where the electrophilic beta carbon is reacted with the thiol group of specific cysteines (Jackson et al., 2017).

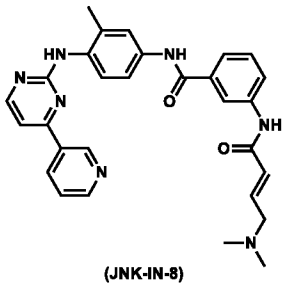
The enhanced and uncontrolled reactivity of the Michael acceptors may lead to adverse (off-target) effects by reacting with undesired proteins containing a cysteine. These unwanted effects may lead to increased idiosyncratic drug reactions and therefore these inhibitors might increase the risk of side-effects. An additional problem is that cells contain free thiol nucleophiles (e.g., glutathione/GSH) in a concentration that is often many orders of magnitude higher than any targeted SH functionality (Krishnan et al., 2014). So, this competitive reactivity may also make electrophilic warheads ineffective, since reduced glutathione is present in millimolar amounts in the cell (e.g., GSH; 1-10 mM) (Meister et al., 1988) (Resnick et al., 2019). Accordingly, significant efforts have been directed to tune the reactivity of Michael acceptors to maintain the adduct forming capacity with the target but minimize/avoid off-target and GSH reactions. Thus, the acrylamide warhead has become widely used to target cysteines because it is weakly electrophilic and requires prior docking of the ligand in the proximity to the cysteine residue for covalent bond formation. While this tempered reactivity mitigated the competitive reaction with GSH, nonreversible covalent bond formation of acrylamides could cause unwanted off-target problems. Thus, reversible inhibitors have been developed which possess more reactive but reversible acrylamide warheads (e.g. cyanoacrylamide) (Serafimova et al., 2012) (Bradshaw et al., 2015). In this modification, however, the enhanced reactivity toward GSH may result in a reduced effective concentration.

As a complementary approach to blocking kinase activity, cellular signaling may also be inhibited by blocking the protein-protein interactions (PPI) of kinases. For example, mitogen-activated protein kinases (MAPKs) have a shallow PPI surface which is used by activators and substrates for binding (Tanoue et al., 2001) (Chang et al., 2002) (Garai et al, 2012) (Glatz et al, 2015). Protein partners all bind to this MAPK D(ocking)-groove by short linear motifs located in their unstructured regions (Zeke et al., 2015). Formerly, there had been several attempts to find small molecules capable of binding in the MAPK D-groove, and thus block MAPK signaling (Hancock et al., 2005) (Stebbins et al., 2008) (Sammons et al., 2019). The D-groove of all MAPKs (e.g., ERK1/2, p38, JNK, ERK5) contain a conserved surface cysteine and covalent modification of this residue may directly interfere with MAPK-partner protein binding. The D-groove has a conserved cysteine in all MAPKs (e.g., C161 in ERK2, C162 in p38α, and C163 in JNK1) and is comprised of a negatively charged area (referred to as the CD groove) and three small hydrophobic pockets (Garai et al., 2012).

Mitogen-activated protein kinases (MAPK) are ubiquitous regulators of cellular physiology. Increased extracellular signal-regulated kinase (ERK1/2) activity is a hallmark in cancer due to its pivotal role in promoting cell growth (Lavoie et al, 2020). Although up-regulation of p38 or c-Jun N-terminal kinase (JNK) activity is also known to be associated with specific cancers, these kinases are better known to be associated with acute or chronic inflammation due to their pivotal role in regulating cell death (Wagner et al, 2009) (Canovas et al, 2021). Moreover, there is increasing clinical relevance for ERK5 signaling in tumor development and progression (Simoes et al, 2016).

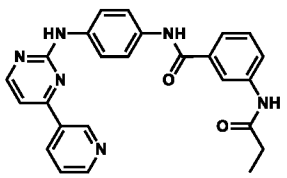
JNKs play critical roles in development and homeostasis and thus complete and sustained JNK inhibition may not be desirable (Bogoyevitch et al, 2008). To date, there are no direct JNK inhibitors approved for use in human therapies. There are three distinct JNK genes (*Jnk1*, *Jnk2*, and *Jnk3*) with about ten different isoforms generated through alternative splicing: the predominant isoforms are JNK1 and JNK2 expressed ubiquitously and JNK3 expressed primarily in the nervous system (Zeke et al., 2016). JNKs are activated by phosphorylation at their activation loop by MAPK kinases (MAP2K) such as MKK4 and MKK7 and are deactivated by MAPK phosphatases including MKP1 and MKP5. JNKs regulate their downstream substrates by phosphorylation. For example, the c-Jun and ATF2 transcription factors are phosphorylated by JNK at Ser63/73 and Thr69/71, respectively. JNKs are activated when cells are exposed to stress conditions such as osmotic stress, hypoxia, UV light, and cytokines and are activated less to exposure to growth factors. Hyperactivation of JNK signaling is common in several disease states including cancer, inflammatory, and neurodegenerative diseases. Based on genetic and pharmacological studies in animal models, JNK inhibitors have anti-inflammatory and neuroprotective effects. Specific blocking of JNK activity could be particularly important in treating diseases such as Alzheimer's disease, Parkinson's disease, asthma, diabetes, and rheumatoid arthritis (Duong et al., 2020). Moreover, selective JNK inhibition would likely have a positive role on neuron regeneration/repair after injury (Shellino et al., 2019). Thus, JNK is an attractive target for therapeutic intervention with small molecule kinase inhibitors. As a result, various inhibitors have been developed; however, limitations are noted with these inhibitors, including lack of specificity and cell toxicity.

To block JNK activity selectively, an ATP binding scaffold – developed from imatinib – was linked with an acrylamide warhead. These compounds selectively target a specific cysteine – conserved in JNK1-3 (cysteine 154 in JNK3, cysteine 116 in JNK1 and JNK2), but unique among MAPKs – through covalent bond formation (Zhang et al., 2012). The inhibitors – where the most selective JNK inhibitor was called JNK-IN-8 (EP 2822935) and will be referred to as such henceforth – were shown to bind to JNK irreversibly. Thus, JNK-IN-8 proved to be a promising JNK-specific kinase inhibitor, nevertheless, the irreversibility of C-S cysteine adducts – formed by an acrylamide warhead – raises concerns about potential unwanted off-target effects and about the half-life in a biological environment (e.g., various S, N nucleophiles, especially 1-10 mM GSH; Meister et al., 1988).



(JNK-IN-8)

Document EP 2822935 also discloses a compound without a covalent warhead (JNK-IN-6), however, this compound exhibited an almost 100-fold less potent biochemical IC_{50} on JNK1, 2, and 3. The structure of the compound differs from the effective compounds in that the acrylamide is replaced with an approximately isosteric propyl amide group.



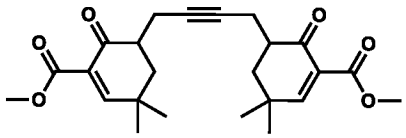
(JNK-IN-6)

The presence of the covalent warhead endows composite drugs with new properties related to the covalent nature of binding (e.g., higher binding affinity and increased residence time). These latter seem to be transferable but the functional parts, namely the noncovalent directing and the covalent warhead moieties, must be linked in such a way that fits with target-specific requirements. The acrylamide warhead, for example, is used repeatedly in different inhibitors binding to various members of highly different kinase families as well as against a specific RAS GTPase mutant harboring the G12C point mutation (Liu et al., 2013) (Krishnan et al., 2014) (Miller et al, 2014) (Zhao et al., 2017) (Chaikuad et al., 2018) (Ostrem et al, 2013) (Kwiatkowski et al, 2014). As an alternative to irreversible covalent drugs, a reversible covalent mechanism can offer a better balance between potency and selectivity compared to irreversible mechanisms (Tuley et al, 2018). Moreover, reversible warheads with low off-rates/long residence time could provide an advantage as parts of proteolysis targeting chimeras (PROTAC) compared to irreversible warheads, because the warhead moiety could be recycled in the cell after target protein degradation (He et al, 2022) (Smith et al, 2019) (Guo et al, 2020) (Ishida et al, 2021).

The covalent modification of histidine residues is inherently more challenging and has been discussed in Jacob C. G. et al. (Jacob et al, 2018), where the design of an inhibitor against wild-type isocitrate dehydrogenase 1 (IDH1) is described acting as an NADPH competitive compound in which an α,β-unsaturated enone 1 makes covalent linkage through active site H315. Furthermore, Joshi P. N. and Rai V. (Joshi et al, 2019) examined C–N bond formation with diverse proteins without adversely affecting their structure and function. While the authors suggested that 2-cyclohexenone is an electrophile that may be applied in various proteins, they do not suggest a possibility of specific targeting approach.

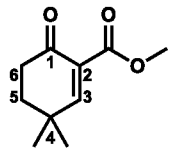
Cyclopentenone or cyclohexenone derivatives had formally been synthesized and used for various synthetic transformations (Berkes et al., 2016) (Varga et al., 2020) (Oswy et al., 1994) (Oswy et al., 1996) (Kato et al., 2002) (Okano et al., 1988) (Dobly et al., 1968) (Maier et al., 1993) (Meyer et al., 2000) (Souza et al, 2002) (Hsieh et al, 2008) (Liu et al, 2005) (Li et al 2022) (Document US11518729B2), however, these studies report only on accidental examples since none of them suggest a possibility of protein targeting. Sauerland M., while analyzing the kinetic aspects of Michael addition reactions of α,β -unsaturated carbonyl compounds to amino acid and protein thiols, noted that cyclopent-2-en-1-one (CPN) and cyclohex-2-en-1-one (CHN) form a stable covalent adduct with soft nucleophiles such as GSH, *N*-acetylcysteine (NAC) and thiols from some model proteins (bovine serum albumin, glyceraldehyde-3-phosphate dehydrogenase, creatine kinase, papain, Keap1) (Sauerland et al, 2021). This study also noted that CHN did not show enhanced reactivity or selectivity towards protein-incorporated cysteines when compared to GSH or NAC. Their measurements with CPN did not give analyzable data and the authors speculated that this may be due to reversibility of the CPN addition reactions, which is of potential biological significance due to the presence of this ring structure in some prostaglandins (Suzuki et al, 1997). These reports, however, do not suggest the usage of these highly unspecific compounds as inhibitors, and do not teach the steric and electronic fine-tuning of such inhibitors. Moreover, based on the findings of Sauerland et al, CHN, due to its better reactivity to GSH, would not be able to function in the nucleophile-rich environment of the cell. Moreover, the data and discussion in Suzuki et al suggest that the apparent antiproliferative effects of cyclopentenone containing prostaglandins are due to irreversible binding to protein thiols, albeit binding to GSH is reversible.

Deny J.L. et al have produced the following bis-Michael acceptor compound:



Deny et al, 2016; 34

which was suggested to be able to target Cys151 in Keap1 and thus increased Nrf2-mediated transcription. The monovalent version was far less potent (Deny et al, 2016; 7).



Deny et al, 2016; 7

The methyl groups at C4 were introduced to increase steric hindrance at this position, because a compound lacking substitutions at C4 were deemed to be too electrophilic, and a dioxolane with two electron withdrawing moieties at this position was found also not useful. Compounds with electronic withdrawing groups at C4 thus were then excluded from their analysis and other electron withdrawing groups (EWG) were not exploited to fine-tune amino acid adduct formation.

Despite the apparent importance and success of covalent warheads in current drug design and developments, the applied warheads display a rather limited structural variance and complexity (e.g., simple open-chain, acyclic acrylamides) which automatically limits the attainable chemical space. Furthermore, to minimize possible side-reactions during the synthesis of drugs, the applied reactive warheads are added appendages in the late-stage of the synthetic route, thus a warhead scaffold that can be synthetically easily varied using orthogonal chemistry and used as a tunable covalent warhead is still missing. Such a structurally more complex scaffold would be much more like the warheads of the natural products and is expected to be more selective in targeting nucleophiles found in proteins. Moreover, owing to a larger contact surface, such a more complex scaffold might be more suitable for targeting shallow protein surfaces involved in protein-protein interactions. Alternatively, more complex warheads, for example cyclic scaffolds, for example with 5- or 6-membered rings, distinct from open-chain acrylamide-based warheads, may also be used as specific anchors linked to bigger directing moieties (noncovalent inhibitors) to further enhance specificity and/or

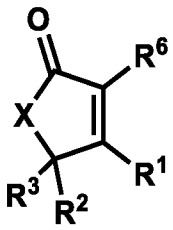
residency time of the composite drug. Briefly, a structurally complex, reversible covalent warhead with the possibility of functionalization at several positions would be useful to increase the binding strength of noncovalent fragments/inhibitors that bind within ~10-20 Å from a targetable amino acid; for example, when the warhead is linked to a noncovalent moiety using an appropriate linker.

Summary of the invention

The basis of the present invention is cyclic, sterically and electronically fine-tuned Michael acceptor-based chemical warheads forming a reversible covalent bond to specific surface amino acids on proteins. Besides the cyclic framework which controls the accessibility of the Michael acceptor, additional critical structural features of these structures are the double activated olefinic group in the α-position and double substitution in γ-position with appropriate electron withdrawing group(s) allowing the fine-tuning of steric and electronic properties. Thus, the special structure of these new warheads allows far better control over the electronic and steric properties influencing covalent bond formation to specific amino acids compared to previously utilized acyclic acrylamides. These tailored warheads mainly target the cysteine, but also other nucleophilic amino acids, e.g., histidine, thus the adduct serves as a covalent anchor capable of increasing the binding affinity and decreasing the off-rate. Furthermore, these reversible warheads provide an alternative to the practice of using an irreversible electrophile, particularly if one considers that unwanted cross-reactions in the cell are likely to be more devastating if happen through irreversible covalent bond formation. The cyclic and chiral nature of the scaffold is another distinct feature offering a further advantage: the local and inherently chiral environment of specific nucleophiles can be better exploited in creating specific inhibitors, for example by varying the configuration of substituent groups within the cyclic warhead scaffold.

The present invention provides compounds of Formula (I), and pharmaceutically acceptable salts and compositions thereof. The present invention further provides methods of using the inventive warhead scaffolds targeting preferably, but not limited to cysteine or histidine, more preferably cysteine, compounds, and pharmaceutically acceptable salts and compositions thereof, to study the inhibition of proteins, by affecting protein levels as parts of proteolysis targeting chimeras (PROTAC) or by affecting protein activity as protein-protein interaction or enzymatic activity inhibitors, particularly kinases, more specifically, but not limited to mitogen-activated protein kinases (MAPKs), and such compounds for use as therapeutics in the prevention and treatment of diseases associated with proteins containing a targetable cysteine or other nucleophilic amino acid, e.g., histidine, for example, but not limited to, kinases or MAPKs. In certain embodiments, the inventive cysteine (or other nucleophilic amino acid, e.g., histidine) modifying warhead scaffolds, compounds are used for the prevention and treatment of proliferative diseases (e.g., cancer), neurodegenerative diseases, metabolic disorders, inflammatory diseases, and cardiovascular diseases.

1. Compound of Formula (I)



Formula (I)

or pharmaceutically acceptable salts thereof
wherein

R^1 is H, C_{1-4} alkyl, C_{2-4} alkenyl, C_{2-4} alkynyl, C_{6-10} aryl or 5-7 membered heteroaryl or perfluorinated C_{1-4} alkyl, preferably H or C_{1-3} alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl, C_{6-10} aryl, more preferably H, C_{1-3} alkyl, or phenyl
or R^1 together with R^2 or R^3 may form a -C(O)-O-$(CH_2)_m$- group where m = 1-2, preferably 1;
or R^1 together with R^2 may form a–-$(CH_2)_n$- where n = 1-5, preferably 3-4;

R^2 is selected from C_{1-4} alkyl, C_{2-4} alkenyl, C_{2-4} alkynyl, C_{3-7} carbocyclyl, 3-7 membered heterocyclyl, C_{6-10} aryl, 5-7 membered heteroaryl, halo-C_{1-4} alkyl, aryl-C_{1-4} alkyl, -C(O)OH, -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{2-4} alkynyl, -C(O)O-C_{6-10} aryl, -C(O)O-$[(CH_2)_2O]_n$-C_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C_{2-4} alkenyl, -C(O)O-$[(CH_2)_2O]_n$-C_{2-4} alkynyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)OH, -C(O)O-$[(CH_2)_2O]_n$-C(O)OC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)O-C_{2-4} alkenyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)O-C_{2-4} alkynyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)NH_2, -C(O)O-$[(CH_2)_2O]_n$-C(O)NHC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)NC_{1-4} alkyl$_2$, -C(O)O-$[(CH_2)_2O]_n$-NH_2, -C(O)O-$[(CH_2)_2O]_n$-NHC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-NC_{1-4} alkyl$_2$, -C(O)O-$[(CH_2)_2O]_n$-C_{1-4} haloalkyl, -C(O)O-$[(CH_2)_2O]_n$-C_{6-10} aryl, -C(O)O-$[(CH_2)_2O]_n$-5-7 membered heteroaryl, -C(O)NH$(CH_2)_n$NHC(O)-C_{6-10} aryl, -C(O)NH$(CH_2)_n$NHC(O)-5-7 membered heteroaryl, -C(O)-C_{1-4} alkyl, -C(O)-C_{2-4} alkenyl, -C(O)-C_{2-4} alkynyl, -C(O)-C_{6-10} aryl, -C_{1-4} alkyl-C(O)-C_{1-4} alkyl, -C_{1-4} alkyl-C(O)-C_{1-4} alkenyl, -C_{1-4} alkyl-C(O)-C_{1-4} alkynyl, -C(O)NH_2, -C(O)NHC_{1-4} alkyl, -C(O)NHC_{2-4} alkenyl, -C(O)NHC_{2-4} alkynyl, -C(O)NC_{1-4} alkyl$_2$, -

$C(O)NC_{2-4}$ alkenyl$_2$, $-C(O)NC_{2-4}$ alkynyl$_2$, -OH, $-OC_{1-4}$ alkyl, $-NH_2$, $-NHC_{1-4}$ alkyl, $-N(C_{1-4}$ alkyl$)_2$, halogen, -CN, $-SO_3H$, $-SO_3C_{1-4}$ alkyl, $-CF_3$, $-C(O)O-C_{1-4}$ alkyl-$C{\equiv}CR^7$, $-C(O)O-C_{1-4}$-alkyl-R^8,
or together with R^5 may form a $-(CH_2)_m-C(CH_2)-$ group,
where n = 1-10
m = 1-2, preferably 1,

R^3 is selected from -C(O)OH, $-C(O)O-C_{1-4}$ alkyl, $-C(O)O-C_{2-4}$ alkenyl, $-C(O)O-C_{2-4}$ alkynyl, $-C(O)O-C_{6-10}$ aryl, $-C(O)O-[(CH_2)_2O]_n-C_{1-4}$ alkyl, $-C(O)O-[(CH_2)_2O]_n-C_{2-4}$ alkenyl, $-C(O)O-[(CH_2)_2O]_n-C_{2-4}$ alkynyl, $-C(O)O-[(CH_2)_2O]_n-C(O)OH$, $-C(O)O-[(CH_2)_2O]_n-C(O)OC_{1-4}$ alkyl, $-C(O)O-[(CH_2)_2O]_n-C(O)O-C_{2-4}$ alkenyl, $-C(O)O-[(CH_2)_2O]_n-C(O)O-C_{2-4}$ alkynyl, $-C(O)O-[(CH_2)_2O]_n-C(O)NH_2$, $-C(O)O-[(CH_2)_2O]_n-C(O)NHC_{1-4}$ alkyl, $-C(O)O-[(CH_2)_2O]_n-C(O)NC_{1-4}$ alkyl$_2$, $-C(O)O-[(CH_2)_2O]_n-NH_2$, $-C(O)O-[(CH_2)_2O]_n-NHC_{1-4}$ alkyl, $-C(O)O-[(CH_2)_2O]_n-NC_{1-4}$ alkyl$_2$, $-C(O)O-[(CH_2)_2O]_n-C_{1-4}$ haloalkyl, $-C(O)O-[(CH_2)_2O]_n-C_{6-10}$ aryl, $-C(O)O-[(CH_2)_2O]_n$-5-7 membered heteroaryl, $-C(O)NH(CH_2)_nNHC(O)-C_{6-10}$ aryl, $-C(O)NH(CH_2)_nNHC(O)$-5-7 membered heteroaryl, $-C(O)-C_{1-4}$ alkyl, $-C(O)-C_{2-4}$ alkenyl, $-C(O)-C_{2-4}$ alkynyl, $-C(O)-C_{6-10}$ aryl, $-C_{1-4}$ alkyl-C(O)-C_{1-4} alkyl, $-C_{1-4}$ alkyl-C(O)-C_{1-4} alkenyl, $-C_{1-4}$ alkyl-C(O)-C_{1-4} alkynyl, $-C(O)NH_2$, $-C(O)NHC_{1-4}$ alkyl, $-C(O)NHC_{2-4}$ alkenyl, $-C(O)NHC_{2-4}$ alkynyl, $-C(O)NC_{1-4}$ alkyl$_2$, $-C(O)NC_{2-4}$ alkenyl$_2$, $-C(O)NC_{2-4}$ alkynyl$_2$, -OH, $-OC_{1-4}$ alkyl, $-NH_2$, $-NHC_{1-4}$ alkyl, $-N(C_{1-4}$ alkyl$)_2$, halogen, -CN, $-SO_3H$, $-SO_3C_{1-4}$ alkyl, $-CF_3$, $-C(O)O-C_{1-4}$ alkyl-$C{\equiv}CR^7$, $-C(O)O-C_{1-4}$-alkyl-R^8,
where n = 1-10 ,
or

R^2 and R^3 may be combined together with the atom to which they are attached to form a C_{4-6} carbocyclyl or 4-6 membered heterocyclyl

X is $-CHR^4-$ or $-CHR^4-CHR^5-$ or direct bond, preferably $-CHR^4-$ or $-CHR^4-CHR^5-$,
wherein

R^4 is H, hydroxyl, C_{1-4} alkyl, C_{2-4} alkenyl,

R^5 is H, C_{1-4} alkyl, C_{2-4} alkenyl, or R^5 together with R^2 may form a–$-(CH_2)_m-C(CH_2)-$ group where m = 1-2, preferably 1,

R^4 and R^5 may be combined together with the atoms to which they are attached to form a C_{3-7} carbocyclyl or 3-7 membered heterocyclyl

R^6 is C_{6-10} aryl, 5-7 membered heteroaryl, halo-C_{1-4} alkyl, aryl-C_{1-4} alkyl, $-C(O)O-C_{1-4}$ alkyl, $-C(O)O-C_{2-4}$ alkenyl, $-C(O)O-C_{2-4}$ alkynyl, $-C(O)O-C_{6-10}$ aryl, $-C(O)-C_{1-4}$ alkyl, $-C(O)O-C_{1-4}$ alkyl-C_{6-10} aryl, $-C(O)-C_{2-4}$ alkenyl, $-C(O)-C_{2-4}$ alkynyl, $-C(O)-C_{6-10}$ aryl, $-C(O)NH_2$, $-C(O)NHC_{1-4}$ alkyl, $-C(O)NHC_{2-4}$ alkenyl, $-C(O)NHC_{2-4}$ alkynyl, $-C(O)NHC_{1-4}$ alkyl-OH, $-C(O)NH-C_{6-10}$ aryl, $-C(O)NH-C_{1-4}$ alkyl-C_{6-10} aryl, $-C(O)NH$-5-10 membered heteroaryl, $-C(O)NC_{1-4}$ alkyl$_2$, $-C(O)NC_{2-4}$ alkenyl$_2$, $-C(O)NC_{2-4}$ alkynyl$_2$, $-SO_3H$, $-SO_3C_{1-4}$ alkyl, $-SO_3C_{6-10}$ aryl, $-SO_2NH_2$, $-SO_2NH$alkyl, $-SO_2NH$aryl, $-CF_3$, $-C(O)O-C_{1-4}$ alkyl-$C{\equiv}CR^7$, $-C(O)O-C_{1-4}$-alkyl-R^8, or
R^6 is composed of 2-10 moieties selected from -C(O)-, -NH-, $-N(CH_3)-$, -O-, $-CH_2-$, optionally substituted $-C_{6-10}$ aryl, optionally substituted 3-7 membered carbocyclyl, optionally substituted 3-7 membered heterocyclyl, optionally substituted 5-7 membered heteroaryl, resulting in a chemically reasonable radical;

R^7 is H, C_{6-10} aryl, 5-7 membered heteroaryl, vinyl, $-[(CH_2)_2O]_n-C_{1-4}$ alkyl, $-[(CH_2)_2O]_n-C_{2-4}$ alkenyl, $-[(CH_2)_2O]_n-C_{2-4}$ alkynyl, $-[(CH_2)_2O]_n-C(O)OH$, $-[(CH_2)_2O]_n-C(O)OC_{1-4}$ alkyl, $-[(CH_2)_2O]_n-C(O)O-C_{2-4}$ alkenyl, $-[(CH_2)_2O]_n-C(O)O-C_{2-4}$ alkynyl, $-[(CH_2)_2O]_n-C(O)NH_2$, $-[(CH_2)_2O]_n-C(O)NHC_{1-4}$ alkyl, $-[(CH_2)_2O]_n-C(O)NC_{1-4}$ alkyl$_2$-$[(CH_2)_2O]_n-NH_2$, $-[(CH_2)_2O]_n-NHC_{1-4}$ alkyl, $-[(CH_2)_2O]_n-NC_{1-4}$ alkyl$_2$, $-[(CH_2)_2O]_n-C_{1-4}$ halogenoalkyl, $-(CH_2)_nC(O)OH$, $-(CH_2)_nC(O)O-C_{1-4}$ alkyl, $-(CH_2)_nC(O)O-C_{2-4}$ alkenyl, $-(CH_2)_nC(O)O-C_{2-4}$ alkynyl, $-(CH_2)_nC(O)NH-C_{1-4}$ alkyl, $-(CH_2)_nC(O)NH-C_{2-4}$ alkenyl, $-(CH_2)_nC(O)NH-C_{2-4}$ alkynyl, $-(CH_2)_nC(O)N-C_{1-4}$ alkyl$_2$, $-(CH_2)_nC(O)N-C_{2-4}$ alkenyl$_2$, $-(CH_2)_nC(O)N-C_{2-4}$ alkynyl$_2$, preferably H, C_{6-10} aryl, 5-7 membered heteroaryl; where the aryl, heteroaryl are in each occurrence optionally substituted with one or more substituents independently selected from C_{1-4} alkyl, halogen, haloalkyl, hydroxyl, $-OC_{1-4}$ alkyl, $-NH_2$, $-NHC_{1-4}$ alkyl, $-N(C_{1-4}$ alkyl$)_2$

R^8 is 5-6-membered heterocyclyl, the heterocyclyl is optionally substituted with one or more substituent independently selected from C_{1-4} alkyl, C_{2-4} alkenyl, C_{2-4} alkynyl, C_{3-10} carbocyclyl, 3-10 membered heterocyclyl, C_{6-10} aryl, 5-10 membered heteroaryl, where the carbocyclyl, heterocyclyl, aryl and heteroaryl are optionally substituted with one or more substituent independently selected from C_{1-4} alkyl, halogen, hydroxyl, $-OC_{1-4}$ alkyl, $-NH_2$, $-NHC_{1-4}$ alkyl, $-N(C_{1-4}$ alkyl$)_2$, or 5-10 membered heteroaryl optionally substituted with 1 or 2 C_{1-2} alkyl; halo-C_{1-4} alkyl, aryl-C_{1-4} alkyl, $-C(O)O-C_{1-4}$ alkyl, $-C(O)O-C_{2-4}$ alkenyl, $-C(O)O-C_{6-10}$ aryl, $-C(O)-C_{1-4}$ alkyl, $-C(O)-C_{2-4}$ alkenyl, $-C(O)-C_{2-4}$ alkynyl, $-C(O)-C_{6-10}$ aryl, $-C(O)-C_{5-10}$ heteroaryl, $-C(O)NH_2$, $-C(O)NHC_{1-4}$ alkyl, $-C(O)NHC_{2-4}$ alkenyl, $-C(O)NHC_{2-4}$ alkynyl, $-C(O)NC_{1-4}$ alkyl$_2$, $-C(O)NC_{2-4}$ alkenyl$_2$, $-C(O)NC_{2-4}$ alkynyl$_2$, -CN, $-SO_3H$, $-SO_3C_{1-4}$ alkyl, $-CF_3$, $-C_{1-4}$ alkyl-C(O)OH, -

C_{1-4} alkyl-C(O)O-C_{1-4} alkyl, -C_{1-4} alkyl-C(O)O-C_{2-4} alkenyl, -C_{1-4} alkyl-C(O)O-C_{2-4} alkynyl, -C_{1-4} alkyl-C(O)NH_2, -C_{1-4} alkyl-C(O)NHC_{1-4} alkyl, -C_{1-4} alkyl-C(O)NC_{1-4} alkyl$_2$, -[$(CH_2)_2O]_n$-C_{1-4} alkyl, -[$(CH_2)_2O]_n$-C_{2-4} alkenyl, -[$(CH_2)_2O]_n$-C_{2-4} alkynyl, -[$(CH_2)_2O]_n$-C(O)OH, -[$(CH_2)_2O]_n$-C(O)OC_{1-4} alkyl, -[$(CH_2)_2O]_n$-C(O)O-C_{2-4} alkenyl, ,-[$(CH_2)_2O]_n$-C(O)O-C_{2-4} alkynyl, -[$(CH_2)_2O]_n$-NH_2, -[$(CH_2)_2O]_n$-NHC_{1-4} alkyl, -[$(CH_2)_2O]_n$-NC_{1-4} alkyl$_2$, -[$(CH_2)_2O]_n$-C_{1-4}halogenoalkyl, -$(CH_2)_n$C(O)OH, -$(CH_2)_n$C(O)O-C_{1-4} alkyl, -$(CH_2)_n$C(O)O-C_{2-4} alkenyl, -$(CH_2)_n$C(O)O-C_{2-4} alkynyl, -$(CH_2)_n$C(O)NH-C_{1-4} alkyl, -$(CH_2)_n$C(O)NH-C_{2-4} alkenyl, -$(CH_2)_n$C(O)NH-C_{2-4} alkynyl, -$(CH_2)_n$C(O)N-C_{1-4} alkyl$_2$, -$(CH_2)_n$C(O)N-C_{2-4} alkenyl$_2$, -$(CH_2)_n$C(O)N-C_{2-4} alkynyl$_2$, -CH_2-C(O)NH$(CH_2)_n$NHC(O)-C_{6-10} aryl, -CH_2-C(O)O$(CH_2)_n$NHC(O)-C_{6-10} aryl, -CH_2-C(O)NH$(CH_2)_n$OC(O)-C_{6-10} aryl, -CH_2-C(O)O$(CH_2)_n$OC(O)-C_{6-10} aryl or -$(CH_2)_n$-C(O)NH-peptide where the peptide sequence consists of 1-20 amino acids, preferably 1-15 amino acids,

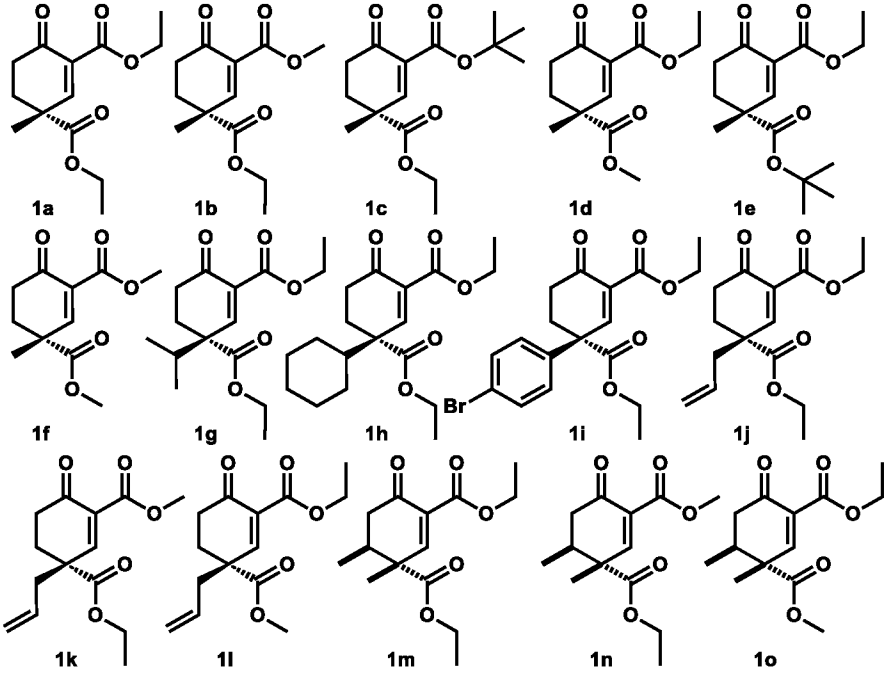
preferably a peptide comprising the sequence of
Xaa_{11} Xaa_{10} Xaa_9 Xaa_8 Xaa_7 Xaa_6 Xaa_5 Xaa_4 Xaa_3 Xaa_2 Xaa_1 *
where * means the *N* terminal of the peptide wherein
Xaa_{11} is an apolar residue, preferably Leu, Ile or Ala, preferably Leu,
Xaa_{10} is an apolar residue, preferably Leu, Ile or Ala, preferably Ala,
Xaa_9 is a residue comprising an amide or a basic residue, preferably Arg or Lys,
Xaa_8 is a residue comprising an amide or a basic residue, preferably Arg or Lys,
Xaa_7 is a residue comprising an amide or a basic residue, preferably Arg or Lys,
wherein at least two of Xaa_9 Xaa_8 Xaa_7 is a basic residue, preferably Arg or Lys,
Xaa_6 is an apolar residue, preferably Leu, Ile or Ala, preferably Ala,
Xaa_5 is an apolar residue, preferably Leu, Ile or Ala, preferably Leu,
Xaa_4 is a residue comprising an amide or a basic residue, preferably Arg or Lys,
Xaa_3 is a polar residue, preferably Ser or Thr,
Xaa_2 is a residue comprising an amide or a basic residue, preferably Arg or Lys,
Xaa_1 is a polar residue, preferably Ser or Thr;

preferably the following sequence: LARRRALRSKS* where * means the N terminal of the peptide

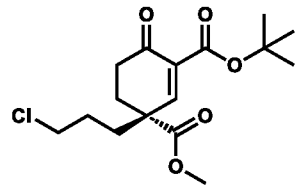
or the substituent of the 5-6-membered heterocyclyl is composed of 2-10 moieties selected from -CH_2-, -C(O)-, -NH-, -$N(CH_3)$-, -O-, optionally substituted -C_{6-10} aryl, optionally substituted 3-7 membered carbocyclyl, optionally substituted 3-7 membered heterocyclyl, optionally substituted 5-7 membered heteroaryl, resulting in a chemically reasonable radical;

wherein
n = 1-10;

1.1 with the proviso that the following compounds are excluded from the claimed scope:
1.1.1 molecules **1a-1o** from Berkes et al., 2016

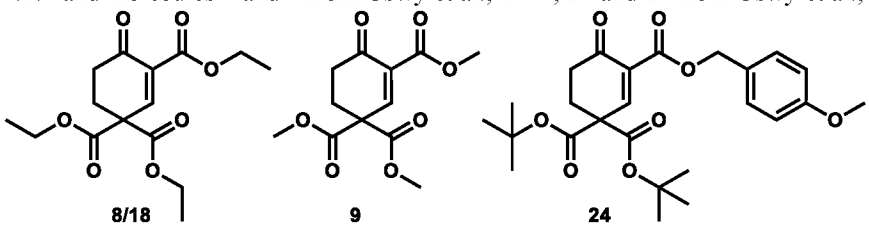


1.1.2 and molecule **7** from Varga et al., 2020



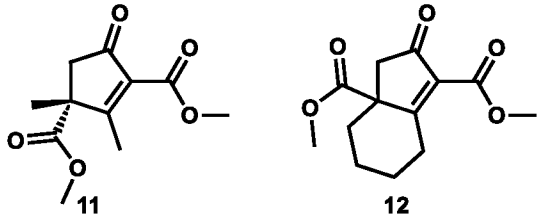
7

1.1.3 and molecules **8** and **9** from Oswy et al., 1994, **18** and **24** from Oswy et al., 1996



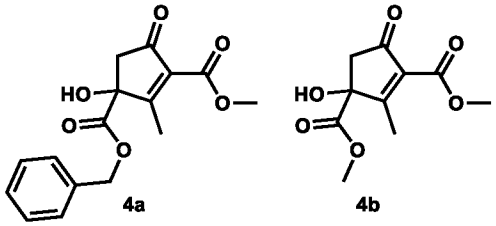
8/18 9 24

1.1.4 and molecules **11** and **12** from Kato et al., 2002



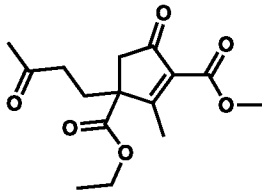
11 12

1.1.5 and molecules **4a/4b** from Okano et al., 1988



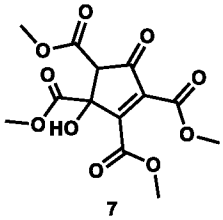
4a 4b

1.1.6 and molecule **9** from Dobly et al., 1968



9

1.1.7 and molecule 7 from Maier et al., 1993



7

2. Compound according to point 1 or pharmaceutically acceptable salts thereof, wherein

R^1 is H, C_{1-4} alkyl, C_{2-4} alkenyl, C_{2-4} alkynyl, C_{6-10} aryl or 5-7 membered heteroaryl, preferably H or C_{1-3} alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl, C_{6-10} aryl, more preferably H, C_{1-3} alkyl, or phenyl;

R^2 is selected from C_{1-4} alkyl, C_{2-4} alkenyl, C_{2-4} alkynyl, C_{3-7} carbocyclyl, 3-7 membered heterocyclyl, C_{6-10} aryl, 5-7 membered heteroaryl, halo-C_{1-4} alkyl, aryl-C_{1-4} alkyl, -C(O)OH, -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{2-4} alkynyl, -C(O)O-C_{6-10} aryl, -C(O)O-$[(CH_2)_2O]_n$-C_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C_{2-4} alkenyl, -C(O)O-$[(CH_2)_2O]_n$-C_{2-4} alkynyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)OH, -C(O)O-$[(CH_2)_2O]_n$-C(O)OC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)O-C_{2-4} alkenyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)O-C_{2-4} alkynyl, -C(O)O-$[(CH_2)_2O]_n$-$C(O)NH_2$, -C(O)O-$[(CH_2)_2O]_n$-C(O)NHC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)NC_{1-4} alkyl$_2$, -C(O)O-$[(CH_2)_2O]_n$-NH_2, -C(O)O-$[(CH_2)_2O]_n$-NHC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-NC_{1-4} alkyl$_2$, -C(O)O-$[(CH_2)_2O]_n$-C_{1-4} haloalkyl, -C(O)O-$[(CH_2)_2O]_n$-C_{6-10} aryl, -C(O)O-$[(CH_2)_2O]_n$-5-7 membered heteroaryl, -C(O)NH$(CH_2)_n$NHC(O) C_{6-10} aryl, -C(O)NH$(CH_2)_n$NHC(O)-5-7 membered heteroaryl, -C(O)-C_{1-4} alkyl, -C(O)-C_{2-4}

alkenyl, -C(O)-C_{2-4} alkynyl, -C(O)-C_{6-10} aryl, -C_{1-4} alkyl-C(O)-C_{1-4} alkyl, -C_{1-4} alkyl-C(O)-C_{1-4} alkenyl, C_{1-4} alkyl-C(O)-C_{1-4} alkynyl, -C(O)NH_2, -C(O)NHC_{1-4} alkyl, -C(O)NHC_{2-4} alkenyl, -C(O)NHC_{2-4} alkynyl, -C(O)NC_{1-4} $alkyl_2$, -C(O)NC_{2-4} $alkenyl_2$, -C(O)NC_{2-4} $alkynyl_2$, -CN, -SO_3H, -SO_3C_{1-4} alkyl, -CF_3, -C(O)O-C_{1-4} alkyl-C≡CR^7, -C(O)O-C_{1-4}-alkyl-R^8,
or together with R^5 may form a -$(CH_2)_m$-$C(CH_2)$- group, where n = 1-10
m = 1-2, preferably 1,

R^3 is selected from -C(O)OH, -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{2-4} alkynyl, -C(O)O-C_{6-10} aryl, -C(O)O-$[(CH_2)_2O]_n$-C_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C_{2-4} alkenyl, -C(O)O-$[(CH_2)_2O]_n$-C_{2-4} alkynyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)OH, -C(O)O-$[(CH_2)_2O]_n$-C(O)OC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)O-C_{2-4} alkenyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)O-C_{2-4} alkynyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)NH_2, -C(O)O-$[(CH_2)_2O]_n$-C(O)NHC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)NC_{1-4} $alkyl_2$, -C(O)O-$[(CH_2)_2O]_n$-NH_2, -C(O)O-$[(CH_2)_2O]_n$-NHC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-NC_{1-4} $alkyl_2$, -C(O)O-$[(CH_2)_2O]_n$-C_{1-4} haloalkyl, -C(O)O-$[(CH_2)_2O]_n$-C_{6-10} aryl, -C(O)O-$[(CH_2)_2O]_n$-5-7 membered heteroaryl, -C(O)$NH(CH_2)_nNHC(O)$ C_{6-10} aryl, -C(O)$NH(CH_2)_nNHC(O)$-5-7 membered heteroaryl, -C(O)-C_{1-4} alkyl, -C(O)-C_{2-4} alkenyl, -C(O)-C_{2-4} alkynyl, -C(O)-C_{6-10} aryl, -C_{1-4} alkyl-C(O)-C_{1-4} alkyl, -C_{1-4} alkyl-C(O)-C_{1-4} alkenyl, C_{1-4} alkyl-C(O)-C_{1-4} alkynyl, -C(O)NH_2, -C(O)NHC_{1-4} alkyl, -C(O)NHC_{2-4} alkenyl, -C(O)NHC_{2-4} alkynyl, -C(O)NC_{1-4} $alkyl_2$, -C(O)NC_{2-4} $alkenyl_2$, -C(O)NC_{2-4} $alkynyl_2$, -CN, -SO_3H, -SO_3C_{1-4} alkyl, -CF_3, -C(O)O-C_{1-4} alkyl-C≡CR^7, -C(O)O-C_{1-4}-alkyl-R^8,
where n = 1-8
or

R^2 and R^3 may be combined together with the atom to which they are attached to form a C_{4-6} carbocyclyl or 4-6 membered heterocyclyl

X is -CHR^4- or -CHR^4-CHR^5- or direct bond, preferably -CHR^4- or -CHR^4-CHR^5-,
wherein

R^4 is H, hydroxyl, C_{1-4} alkyl, C_{2-4} alkenyl,

R^5 is H, C_{1-4} alkyl, C_{2-4} alkenyl, or R^5 together with R^3 may form a –$(CH_2)_m$-$C(CH_2)$- group where m = 1-2, preferably 1,

R^4 and R^5 may be combined together with the atoms to which they are attached to form a C_{3-7} carbocyclyl or 3-7 membered heterocyclyl

R^6 is C_{3-7} carbocyclyl, 3-7 membered heterocyclyl, C_{6-10} aryl, 5-7 membered heteroaryl, halo-C_{1-4} alkyl, aryl-C_{1-4} alkyl, -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{2-4} alkynyl, -C(O)O-C_{6-10} aryl, -C(O)-C_{1-4} alkyl, -C(O)O-C_{1-4} alkyl-C_{6-10} aryl, -C(O)-C_{2-4} alkenyl, -C(O)-C_{2-4} alkynyl, -C(O)-C_{6-10} aryl, -C(O)NH_2, -C(O)NHC_{1-4} alkyl, -C(O)NHC_{2-4} alkenyl, -C(O)NHC_{2-4} alkynyl, -C(O)NHC_{1-4} alkyl-OH, -C(O)NH-C_{6-10} aryl, -C(O)NH-C_{1-4} alkyl-C_{6-10} aryl, -C(O)NH-5-10 membered heteroaryl, -C(O)NC_{1-4} $alkyl_2$, -C(O)NC_{2-4} $alkenyl_2$, -C(O)NC_{2-4} $alkynyl_2$, -SO_3H, -SO_3C_{1-4} alkyl, -SO_3C_{6-10} aryl, -CF_3, -C(O)O-C_{1-4} alkyl-C≡CR^7 , -C(O)O-C_{1-4}-alkyl-R^8, or
R^6 is composed of 2-10 moieties selected from -C(O)-, -NH-, -$N(CH_3)$-, -O-, -CH_2- optionally substituted -C_{6-10} aryl, optionally substituted 3-7 membered carbocyclyl, optionally substituted 3-7 membered heterocyclyl, optionally substituted 5-7 membered heteroaryl, resulting in a chemically reasonable radical;

R^7 is H, C_{6-10} aryl, 5-7 membered heteroaryl, vinyl, -$[(CH_2)_2O]_n$-C_{1-4} alkyl, -$[(CH_2)_2O]_n$-C_{2-4} alkenyl, -$[(CH_2)_2O]_n$-C_{2-4} alkynyl, -$[(CH_2)_2O]_n$-C(O)OH, -$[(CH_2)_2O]_n$-C(O)OC_{1-4} alkyl, -$[(CH_2)_2O]_n$-C(O)O-C_{2-4} alkenyl, -$[(CH_2)_2O]_n$-C(O)O-C_{2-4} alkynyl, -$[(CH_2)_2O]_n$-C(O)NH_2, -$[(CH_2)_2O]_n$-C(O)NHC_{1-4} alkyl, -$[(CH_2)_2O]_n$-C(O)NC_{1-4} $alkyl_2$-$[(CH_2)_2O]_n$-NH_2, -$[(CH_2)_2O]_n$-NHC_{1-4} alkyl, -$[(CH_2)_2O]_n$-NC_{1-4} $alkyl_2$, -$[(CH_2)_2O]_n$-C_{1-4} halogenoalkyl, -$(CH_2)_n$C(O)OH, -$(CH_2)_n$C(O)O-C_{1-4} alkyl, -$(CH_2)_n$C(O)O-C_{2-4} alkenyl, -$(CH_2)_n$C(O)O-C_{2-4} alkynyl, -$(CH_2)_n$C(O)NH-C_{1-4} alkyl, -$(CH_2)_n$C(O)NH-C_{2-4} alkenyl, -$(CH_2)_n$C(O)NH-C_{2-4} alkynyl, -$(CH_2)_n$C(O)N-C_{1-4} $alkyl_2$, -$(CH_2)_n$C(O)N-C_{2-4} $alkenyl_2$, -$(CH_2)_n$C(O)N-C_{2-4} $alkynyl_2$, preferably H, C_{6-10} aryl, 5-7 membered heteroaryl; where the aryl, heteroaryl are in each occurrence optionally substituted with one or more substituents independently selected from C_{1-4} alkyl, halogen, hydroxyl, -OC_{1-4} alkyl, -NH_2, -NHC_{1-4} alkyl, -$N(C_{1-4}$ alkyl$)_2$

R^8 is 5-6-membered heterocyclyl, the heterocyclyl is optionally substituted with one or more substituent independently selected from C_{1-4} alkyl, C_{2-4} alkenyl, C_{2-4} alkynyl, C_{3-10} carbocyclyl, 3-10 membered heterocyclyl, C_{6-10} aryl, 5-10 membered heteroaryl, the carbocyclyl, heterocyclyl, aryl and heteroaryl are optionally substituted with one or more substituent independently selected from C_{1-4} alkyl, halogen, hydroxyl, -OC_{1-4} alkyl, -NH_2, -NHC_{1-4} alkyl, -$N(C_{1-4}$ alkyl$)_2$or 5-10 membered heteroaryl; halo-C_{1-4} alkyl, aryl-C_{1-4} alkyl, -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{6-10} aryl, -C(O)-C_{1-4} alkyl, -C(O)-C_{2-4} alkenyl, -C(O)-C_{2-4} alkynyl, -C(O)-C_{6-10} aryl, -C(O)-C_{5-10} heteroaryl, -C(O)NH_2, -C(O)NHC_{1-4} alkyl, -C(O)NHC_{2-4} alkenyl, -C(O)NHC_{2-4} alkynyl, -C(O)NC_{1-4} $alkyl_2$, -C(O)NC_{2-4} $alkenyl_2$, -C(O)NC_{2-4} $alkynyl_2$, -CN, -SO_3H, -SO_3C_{1-4} alkyl, -CF_3, -C_{1-4} alkyl-C(O)OH, -C_{1-4} alkyl-C(O)O-C_{1-4} alkyl, -C_{1-4} alkyl-C(O)O-C_{2-4}

$_4$alkenyl, -C_{1-4} alkyl-C(O)O-C_{2-4}alkynyl, -C_{1-4}alkyl-C(O)NH_2, -C_{1-4} alkyl-C(O)NHC_{1-4} alkyl, -C_{1-4} alkyl-C(O)NC_{1-4} alkyl$_2$, -[$(CH_2)_2O$]$_n$-C_{1-4} alkyl, -[$(CH_2)_2O$]$_n$-C_{2-4} alkenyl, -[$(CH_2)_2O$]$_n$-C_{2-4} alkynyl, -[$(CH_2)_2O$]$_n$-C(O)OH, -[$(CH_2)_2O$]$_n$-C(O)OC_{1-4} alkyl, -[$(CH_2)_2O$]$_n$-C(O)O-C_{2-4} alkenyl, -[$(CH_2)_2O$]$_n$-C(O)O-C_{2-4} alkynyl, -[$(CH_2)_2O$]$_n$-NH_2, -[$(CH_2)_2O$]$_n$-NHC_{1-4} alkyl, -[$(CH_2)_2O$]$_n$-NC_{1-4} alkyl$_2$, -[$(CH_2)_2O$]$_n$-C_{1-4} halogenoalkyl, -$(CH_2)_n$C(O)OH, -$(CH_2)_n$C(O)O-C_{1-4} alkyl, -$(CH_2)_n$C(O)O-C_{2-4} alkenyl, -$(CH_2)_n$C(O)O-C_{2-4} alkynyl, -$(CH_2)_n$C(O)NH-C_{1-4} alkyl, -$(CH_2)_n$C(O)NH-C_{2-4} alkenyl, -$(CH_2)_n$C(O)NH-C_{2-4} alkynyl, -$(CH_2)_n$C(O)N-C_{1-4} alkyl$_2$, -$(CH_2)_n$C(O)N-C_{2-4} alkenyl$_2$, -$(CH_2)_n$C(O)N-C_{2-4} alkynyl$_2$, -CH_2-C(O)NH$(CH_2)_n$NHC(O)-C_{6-10} aryl, -CH_2-C(O)O$(CH_2)_n$NHC(O)-C_{6-10} aryl, -CH_2-C(O)NH$(CH_2)_n$OC(O)-C_{6-10} aryl, -CH_2-C(O)O$(CH_2)_n$OC(O)-C_{6-10} aryl
or -$(CH_2)_n$-C(O)NH-peptide where the peptide sequence consists of 1-13 amino acids,
wherein
n = 1-8,
preferably a sequence of
Xaa_{11} Xaa_{10} Xaa_9 Xaa_8 Xaa_7 Xaa_6 Xaa_5 Xaa_4 Xaa_3 Xaa_2 Xaa_1 *
where * means the *N* terminal of the peptide wherein
Xaa_{11} is Leu, Ile or Ala, preferably Leu,
Xaa_{10} is Leu, Ile or Ala, preferably Ala,
Xaa_9 is Arg or Lys,
Xaa_8 is Arg or Lys,
Xaa_7 is Arg or Lys,
Xaa_6 is Leu, Ile or Ala, preferably Ala,
Xaa_5 is Leu, Ile or Ala, preferably Leu,
Xaa_4 is Arg or Lys,
Xaa_3 is Ser or Thr,
Xaa_2 is Arg or Lys,
Xaa_1 is Ser or Thr;
preferably the following sequence: LARRRALRSKS* where * means the N terminal of the peptide.

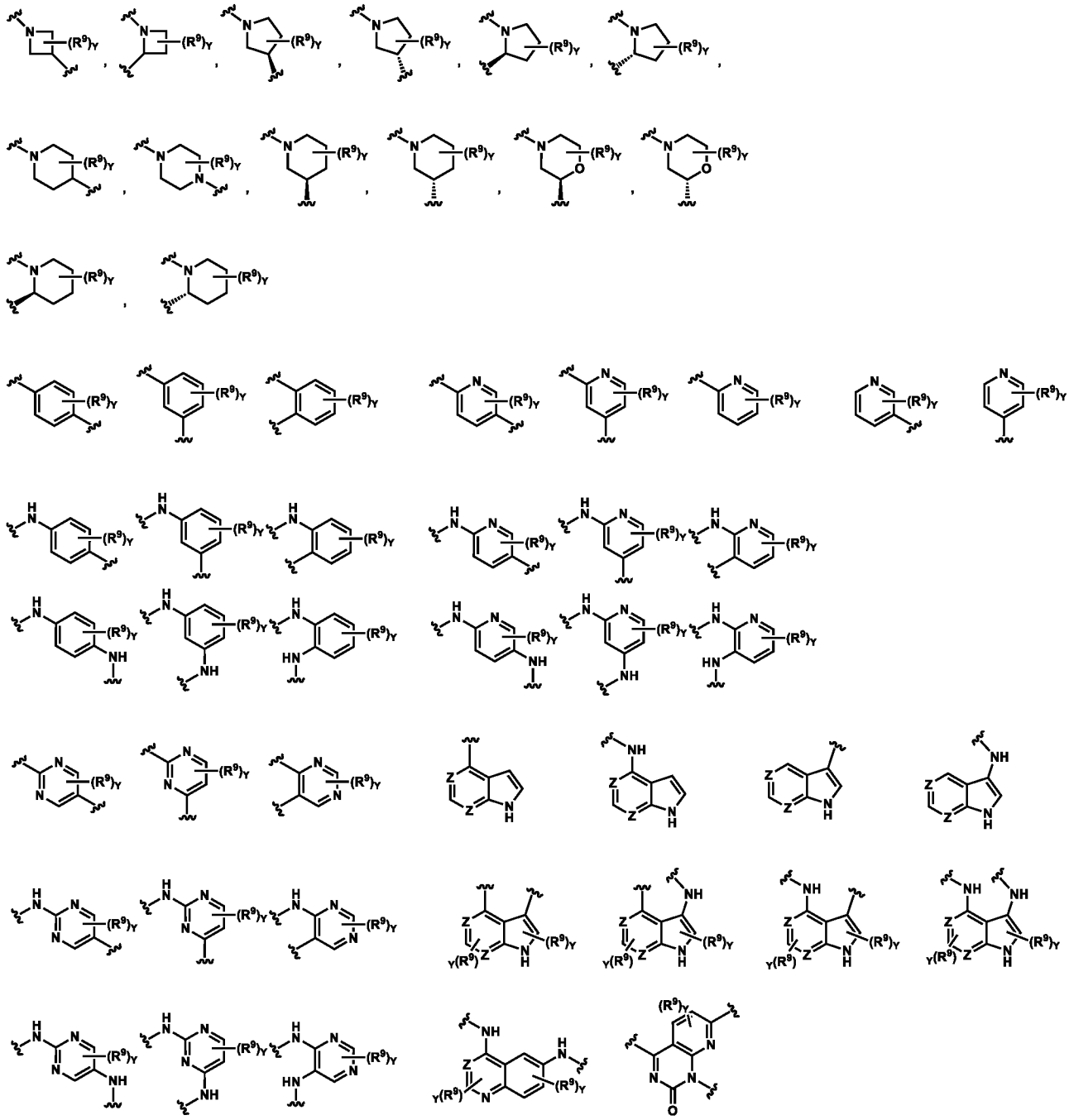
3. Compound according to point 2 or pharmaceutically acceptable salts thereof, wherein

R^1 is H, C_{1-4} alkyl, C_{6-10} aryl, preferably H, C_{1-3} alkyl, phenyl, most preferably H

R^2 is selected from C_{1-4} alkyl, C_{2-4} alkenyl, C_{2-4} alkynyl, C_{3-7} carbocyclyl, 3-7 membered heterocyclyl, C_{6-10} aryl, 5-7 membered heteroaryl, halo-C_{1-4} alkyl, aryl-C_{1-4} alkyl, -C(O)OH, -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{2-4} alkynyl, -C(O)O-C_{6-10} aryl, -C(O)O-[$(CH_2)_2O$]$_n$-C(O)OH, -C(O)O-[$(CH_2)_2O$]$_n$-C(O)OC_{1-4} alkyl, -C(O)O-[$(CH_2)_2O$]$_n$-C(O)NH_2, -C(O)O-[$(CH_2)_2O$]$_n$-C(O) NHC_{1-4} alkyl, -C(O)O-[$(CH_2)_2O$]$_n$-C(O) NC_{1-4} alkyl$_2$, -C(O)O-[$(CH_2)_2O$]$_n$-NH_2, -C(O)O-[$(CH_2)_2O$]$_n$-NHC_{1-4} alkyl, -C(O)O-[$(CH_2)_2O$]$_n$-NC_{1-4} alkyl$_2$, -C(O)O-[$(CH_2)_2O$]$_n$- C_{6-10} aryl, -C(O)O-[$(CH_2)_2O$]$_n$-5-7 membered heteroaryl, -C(O)O-[$(CH_2)_2O$]$_n$-C_{1-4} alkynyl, -C(O)NH$(CH_2)_n$NHC(O) C_{6-10} aryl, -C(O)NH$(CH_2)_n$NHC(O)-5-7 membered heteroaryl, -C(O)-C_{1-4} alkyl, -C(O)-C_{2-4} alkenyl, -C(O)-C_{2-4} alkynyl, -C(O)-C_{6-10} aryl, -C(O)O-C_{1-4} alkyl-C≡CR^7, -C(O)O-C_{1-4}-alkyl-R^8, or together with R^5 may form a -$(CH_2)_m$-C(CH_2)- group, wherein
n = 1-6
m = 1-2, preferably 1;

R^3 is selected from -C(O)OH, -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{2-4} alkynyl, -C(O)O-C_{6-10} aryl, -C(O)O-[$(CH_2)_2O$]$_n$-C(O)OH, -C(O)O-[$(CH_2)_2O$]$_n$-C(O)OC_{1-4} alkyl, -C(O)O-[$(CH_2)_2O$]$_n$-C(O)NH_2, -C(O)O-[$(CH_2)_2O$]$_n$-C(O) NHC_{1-4} alkyl, -C(O)O-[$(CH_2)_2O$]$_n$-C(O) NC_{1-4} alkyl$_2$, -C(O)O-[$(CH_2)_2O$]$_n$-NH_2, -C(O)O-[$(CH_2)_2O$]$_n$-NHC_{1-4} alkyl, -C(O)O-[$(CH_2)_2O$]$_n$-NC_{1-4} alkyl$_2$, -C(O)O-[$(CH_2)_2O$]$_n$- C_{6-10} aryl, -C(O)O-[$(CH_2)_2O$]$_n$-5-7 membered heteroaryl, -C(O)O-[$(CH_2)_2O$]$_n$-C_{1-4} alkynyl, -C(O)NH$(CH_2)_n$NHC(O) C_{6-10} aryl, -C(O)NH$(CH_2)_n$NHC(O)-5-7 membered heteroaryl, -C(O)-C_{1-4} alkyl, -C(O)-C_{2-4} alkenyl, -C(O)-C_{2-4} alkynyl, -C(O)-C_{6-10} aryl, -C(O)O-C_{1-4} alkyl-C≡CR^7, -C(O)O-C_{1-4}-alkyl-R^8, wherein
n = 1-6

R^6 is 3-7 membered heterocyclyl, C_{6-10} aryl, 5-7 membered heteroaryl, -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{2-4} alkynyl, -C(O)O-C_{6-10} aryl, -C(O)O-C_{1-4} alkyl-C_{6-10} aryl, -C(O)NH_2, -C(O)NHC_{1-4} alkyl, -C(O)NHC_{2-4} alkenyl, -C(O)NHC_{2-4} alkynyl, -C(O)NC_{1-4} alkyl$_2$, -C(O)NC_{2-4} alkenyl$_2$, -C(O)NC_{2-4} alkynyl$_2$, -C(O)NHC_{1-4} alkyl-OH, -C(O)NH-C_{1-4} alkyl-C_{6-10} aryl, -C(O)NH-5-10 membered heteroaryl, -C(O)O-C_{1-4} alkyl-C≡CR^7, -C(O)O-C_{1-4}-alkyl-R^8, or
R^6 is composed of 2-10 moieties selected from -C(O)-, -NH-, -N(CH_3)-, -O-, -CH_2-,



wherein

R^9 is -OH, $-NH_2$, $-OC_{1-4}$ Alkyl, $-C_{1-4}$ Alkyl, Halogen, -CN

Y is 0-3, preferably 0-1

Z is CH, N or NH as valency permits

X is $-CHR^4-$ or $-CHR^4-CHR^5-$ or direct bond, preferably $-CHR^4-CHR^5-$

wherein

R^4 is H, C_{1-3} alkyl, C_{2-4} alkenyl, preferably H or C_{1-3} alkyl

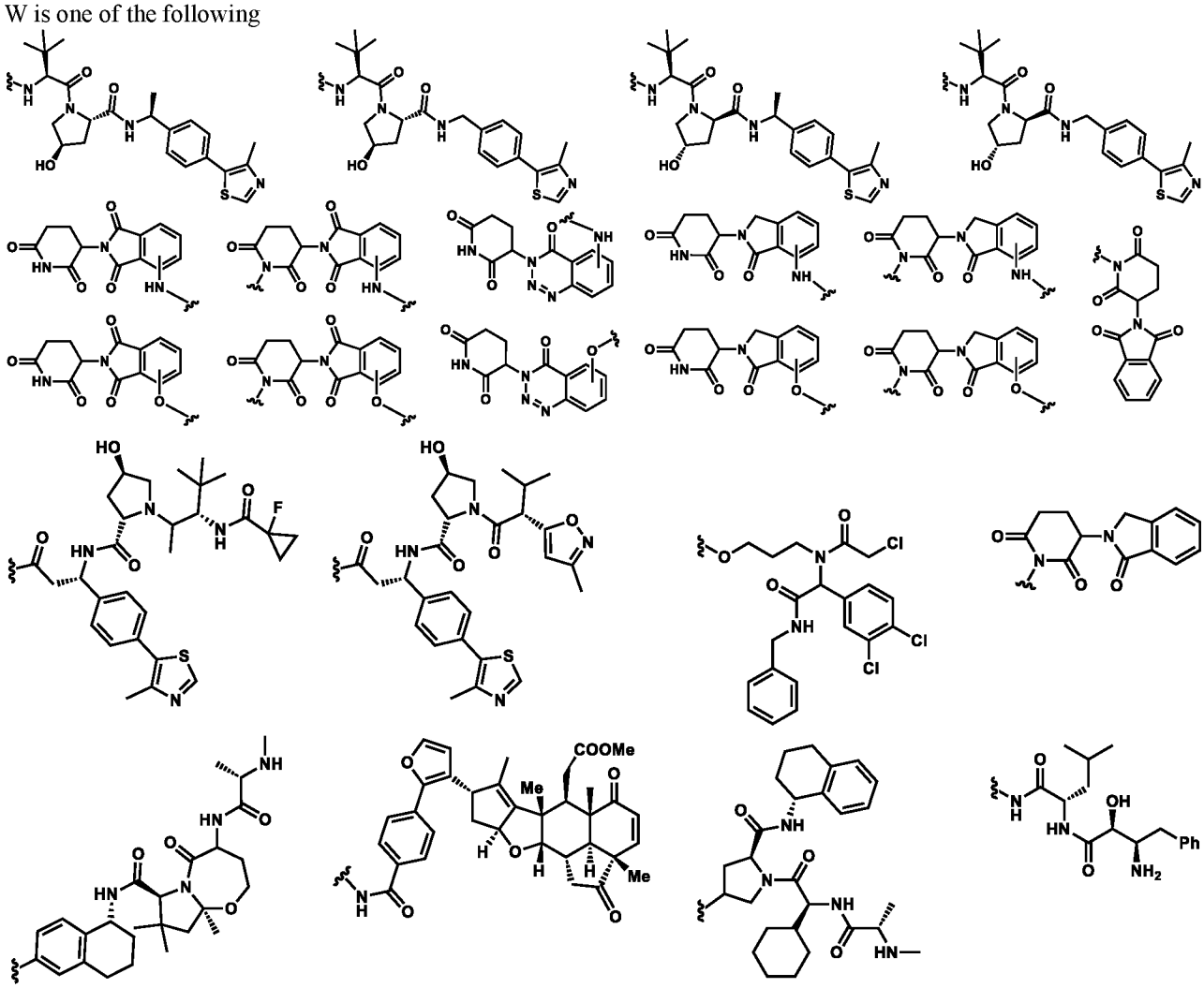
R^5 is H, C_{1-3} alkyl, C_{2-4} alkenyl, or R^5 together with R^3 may form a $-CH_2-C(CH_2)-$ group

R^7 is H, C_{6-10} aryl, 5-7 membered heteroaryl, the aryl and heteroaryl are optionally substituted with one or more substituent independently selected from C_{1-4} alkyl, halogen, $-NH_2$, $-NHC_{1-4}$ alkyl, $-N(C_{1-4}$ alkyl$)_2$; vinyl, $-[(CH_2)_2O]_n-C(O)OH$, $-[(CH_2)_2O]_n-C(O)OC_{1-4}$ alkyl, $-[(CH_2)_2O]_n-C(O)NH_2$, $-[(CH_2)_2O]_n-C(O)NHC_{1-4}$ alkyl, $-[(CH_2)_2O]_n-C(O)NC_{1-4}$ alkyl$_2$-$[(CH_2)_2O]_n-NH_2$, $-[(CH_2)_2O]_n-NHC_{1-4}$ alkyl, $-[(CH_2)_2O]_n-NC_{1-4}$ alkyl$_2$, $-C(O)O-[(CH_2)_2O]_n-$ C_{6-10} aryl, -C(O)O-

$[(CH_2)_2O]_n$-5-7 membered heteroaryl, -C(O)NH$(CH_2)_n$NHC(O) C_{6-10} aryl, -C(O)NH$(CH_2)_n$NHC(O)-5-7 membered heteroaryl, preferably H, C_{6-10} aryl, 5-7 membered heteroaryl

R^8 is 5-6-membered heterocyclyl, the heterocyclyl is optionally substituted with one or more substituent independently selected from C_{1-4} alkyl, C_{2-4} alkenyl, C_{2-4} alkynyl, C_{3-6} carbocyclyl, 3-6 membered heterocyclyl, C_{6-8} aryl, 5-10 membered heteroaryl, where the carbocyclyl, heterocyclyl, aryl and heteroaryl are optionally substituted with one or more substituent independently selected from C_{1-4} alkyl, halogen, hydroxyl, -OC_{1-4} alkyl, -NH_2, -NHC_{1-4} alkyl, -N(C_{1-4} alkyl)$_2$ or 5-10 membered heteroaryl;
halo-C_{1-4} alkyl, aryl-C_{1-4} alkyl, -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{6-10} aryl, -C(O)-C_{1-4} alkyl, -C(O)-C_{2-4} alkenyl, -C(O)-C_{2-4} alkynyl, -C(O)-C_{6-10} aryl, -C(O)NH_2, -C(O)NHC_{1-4} alkyl, -C(O)NHC_{2-4} alkenyl, -C(O)NHC_{2-4} alkynyl, -C(O)NC_{1-4} alkyl$_2$, -C(O)NC_{2-4} alkenyl$_2$, -C(O)NC_{2-4} alkynyl$_2$, -CN, -SO_3H, -SO_3C_{1-4} alkyl, -CF_3, -C_{1-4} alkyl-C(O)O-C_{1-4} alkyl, -C_{1-4} alkyl-C(O)OH, -C_{1-4} alkyl-C(O)O-C_{2-4}alkenyl, -C_{1-4}alkyl-C(O)NH_2, -C_{1-4} alkyl-C(O)NH_2, -C_{1-4} alkyl-C(O)NHC_{1-4} alkyl, -C_{1-4} alkyl-C(O)NC_{1-4} alkyl$_2$, -$[(CH_2)_2O]_n$-C(O)OH, -$[(CH_2)_2O]_n$-C(O)OC_{1-4} alkyl, -$[(CH_2)_2O]_n$-NH_2, -$[(CH_2)_2O]_n$-NHC_{1-4} alkyl, -$[(CH_2)_2O]_n$-NC_{1-4} alkyl$_2$, -CH_2-C(O)NH$(CH_2)_n$NHC(O)- C_{6-10} aryl, -CH_2-C(O)O$(CH_2)_n$NHC(O)-C_{6-10} aryl, -CH_2-C(O)NH$(CH_2)_n$OC(O)-C_{6-10} aryl, -CH_2-C(O)O$(CH_2)_n$OC(O)-C_{6-10} aryl, C(O)NH$(CH_2)_n$NHC(O)-5-7 membered heteroaryl
or -$(CH_2)_n$-C(O)NH-peptide where the peptide sequence consists of 1-12 amino acids, preferably the following sequence: LARRRALRSKS* where * means the N terminal of the peptide
wherein
n = 1-6; or
R^8 is 5-6-membered heterocyclyl, the heterocyclyl is optionally substituted with -$[(CH_2)_2O]_n$-C(O)W , -$[(CH_2)_2O]_n$NHC(O)CH_2W, -CH_2C(O)NH$(CH_2)_n$C(O)W wherein
n = 1-6 and

W is one of the following



4. Compound according to any of the previous points or pharmaceutically acceptable salts thereof, wherein R^1 is H or methyl, C_6 aryl, preferably H.

5. Compound according to any of the previous points or pharmaceutically acceptable salts thereof, wherein R^2 is selected from C_{1-3} alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl; carbocycle selected from C_{3-6} cycloalkyl groups, preferably cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl; heterocycle selected from 3-7 membered heterocyclyl, preferably 4-6 membered heterocyclyl, more preferably azetidinyl, oxetanyl, pyrrolidinyl, tetrahydrofuranyl, piperidinyl, pyranyl, morpholinyl, oxazinyl, dioxanyl; C_{6-10} aryl, 5-7 membered heteroaryl, halo-C_{1-3}alkyl, C_{6-10} aryl-C_{1-3}alkyl; -C(O)O-C_{1-4}alkyl, -C(O)O-C_{2-3}alkenyl, -C(O)O-C_{1-4} alkyl-C≡CR^7, -C(O)O-C_{1-3} alkyl-R^8, -C(O)O-C_{6-10} aryl, -C(O)-C_{1-3} alkyl, -C(O)-C_{2-3} alkenyl, -C(O)-C_{2-3} alkynyl, -C(O)-C_6 aryl,
or together with R^5 may form a -CH_2-C(CH_2)- group,

R^3 is selected from -C(O)O-C_{1-4}alkyl, -C(O)O-C_{2-3}alkenyl, -C(O)O-C_{1-4} alkyl-C≡CR^7, -C(O)O-C_{1-3} alkyl-R^8, -C(O)O-C_{6-10} aryl, -C(O)-C_{1-3} alkyl, -C(O)-C_{2-3} alkenyl, -C(O)-C_{2-3} alkynyl, -C(O)-C_6 aryl,
and

R^7 is H, C_{6-8} aryl, 5-6 membered heteroaryl, the aryl, heteroaryl optionally substituted with one or more substituents independently selected from C_{1-2} alkyl, halogen, hydroxyl, -OC_{1-4} alkyl, -NH_2, -NHC_{1-2} alkyl, -N(C_{1-2} alkyl)$_2$

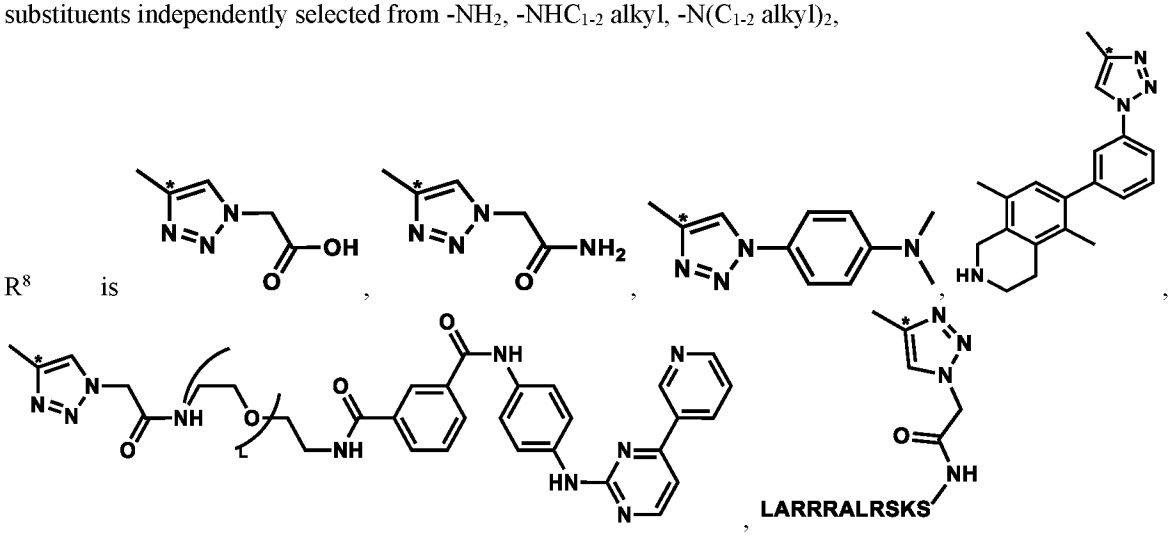
R^8 is 5-6-membered heteroaryl, preferably the heteroaryl comprises 1-3 N atoms, the heteroaryl is optionally substituted with C_6 aryl, the aryl is optionally substituted with one or more substituent independently selected from C_{1-2} alkyl, halogen, hydroxyl, -OC_{1-4} alkyl, -NH_2, -NHC_{1-2} alkyl, -N(C_{1-2} alkyl)$_2$ or 5-10 membered heteroaryl; C_{1-3} alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl, halo-C_{1-3}alkyl, -C(O)NH_2, -C(O)NHC_{1-3} alkyl, -C(O)NC_{1-3} alkyl$_2$, -C_{1-3} alkyl-C(O)O-C_{1-3} alkyl, -C_{1-3} alkyl-C(O)OH, -C_{1-3} alkyl-C(O)O-C_{2-3} alkenyl, -C_{1-3} alkyl-C(O)NH_2, -C_{1-3} alkyl-C(O)NHC_{1-3} alkyl, -C_{1-3} alkyl-C(O)NC_{1-3} alkyl$_2$ or -(CH_2)$_n$-C(O)NH-peptide where the peptide sequence consists of 1-11 amino acids, preferably the following sequence: LARRRALRSKS* where * means the N terminal of the peptide

6. Compound according to any of the previous points or pharmaceutically acceptable salts thereof, wherein

R^2 is selected from methyl, cyclohexyl, phenyl, -CH_2-phenyl, halo-C_{1-3} alkyl, -CH_2-CH_2-CH_2Cl, -CH_2-CH=CH_2, 4-Br-phenyl, -C(O)O-methyl, -C(O)O-ethyl, -C(O)O-*tert*butyl, -C(O)-phenyl, -C(O)O-CH_2-C≡CR^7, -C(O)O-(CH_2)$_2$-C≡CR^7, -C(O)O-(CH_2)$_3$-C≡CR^7, -C(O)O-(CH_2)$_4$-C≡CR^7, or -C(O)O-CH_2-R^8,

R^3 is selected from -C(O)O-methyl, -C(O)O-ethyl, -C(O)O-*tert*butyl, -C(O)-phenyl, -C(O)O-CH_2-C≡CR^7, -C(O)O-(CH_2)$_2$-C≡CR^7, -C(O)O-(CH_2)$_3$-C≡CR^7, -C(O)O-(CH_2)$_4$-C≡CR^7, or -C(O)O-CH_2-R^8

wherein
R^7 is H, C_{6-10} aryl, 5-6 membered heteroaryl, the aryl, heteroaryl optionally substituted with one or more substituents independently selected from -NH_2, -NHC_{1-2} alkyl, -N(C_{1-2} alkyl)$_2$,

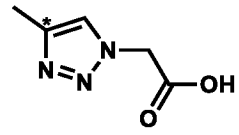
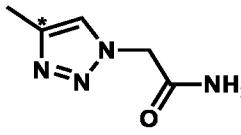
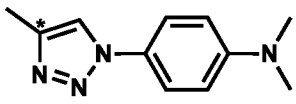
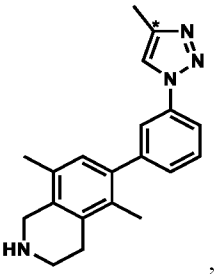
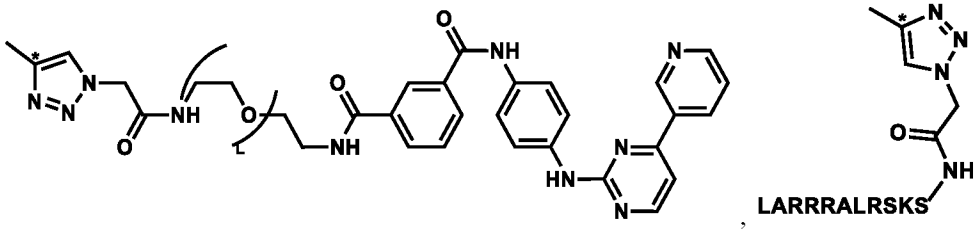
R^8 is 

where * is the point of attachment and
L = 1-6
or R^2 together with R^5 may form a -CH_2-C(CH_2)- group.

7. Compound according to any of points 1-6 or pharmaceutically acceptable salts thereof, wherein

R^2 is C_{1-3} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{3-6} carbocyclyl, 3-6 membered heterocyclyl, C_6 aryl, 5-7 membered heteroaryl, halo-C_{1-3} alkyl, aryl-C_{1-3} alkyl, or together with R^5 may form a -CH_2-C(CH_2)- group

R^3 is -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{2-4} alkynyl, -C(O)O-C_{6-10} aryl, -C(O)-C_{1-3} alkyl, -C(O)-C_{2-4} alkenyl, -C(O)-C_{2-4} alkynyl, -C(O)-C_{6-10} aryl, -C(O)NH_2, -C(O)O-C_{1-4} alkyl-C≡CR^7, -C(O)O-C_{1-4} alkyl-R^8, wherein R^7 is H, C_{6-10} aryl, 5-6 membered heteroaryl, preferably H, C_{6-10} aryl, the aryl is optionally substituted with one or more substituent independently selected from C_{1-2} alkyl, halogen, hydroxyl, -NH_2, -NHC_{1-4} alkyl, -N(C_{1-4} alkyl)$_2$;

and R^8 is  ,  ,  ,  ,  , where * is the point of attachment and
L = 1-3

X is -CHR^4- or -CHR^4-CHR^5-,
wherein
R^4 is H, methyl,

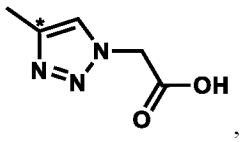
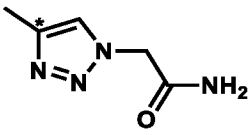
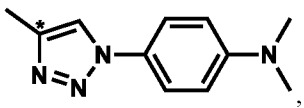
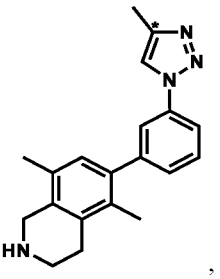
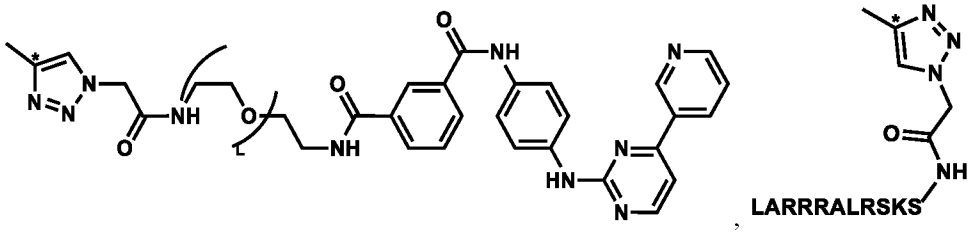
R^5 is H, or together with R^2 may form a -CH_2-C(CH_2)- group

8. Compound according to any of the previous points or pharmaceutically acceptable salts thereof, wherein

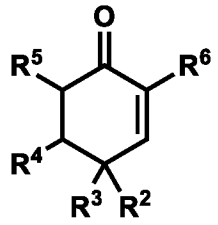
R^2 is methyl, cyclohexyl, phenyl, -CH_2-phenyl, -CH_2-CH_2-CH_2Cl, -CH_2-CH=CH_2, 4-Br-phenyl, or together with R^5 may form a -CH_2-C(CH_2)- group, and

R^3 is -C(O)O-methyl, -C(O)O-ethyl, -C(O)O-*tert*-butyl, -C(O)-phenyl, -C(O)O-CH_2-C≡CR^7, or -C(O)O-CH_2-R^8, wherein

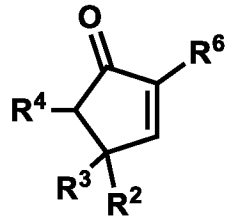
R^7 is H, 4-pyridyl, phenyl, optionally substituted with -NMe_2

R^8 is  ,  ,  ,  ,  ,
where * is the point of attachment and
L = 1-3.

9. Compound according to any of the previous points or pharmaceutically acceptable salts thereof, wherein the compound of formula (I) is a compound of formula (I-1) or formula (I-2)



Formula (I-1)

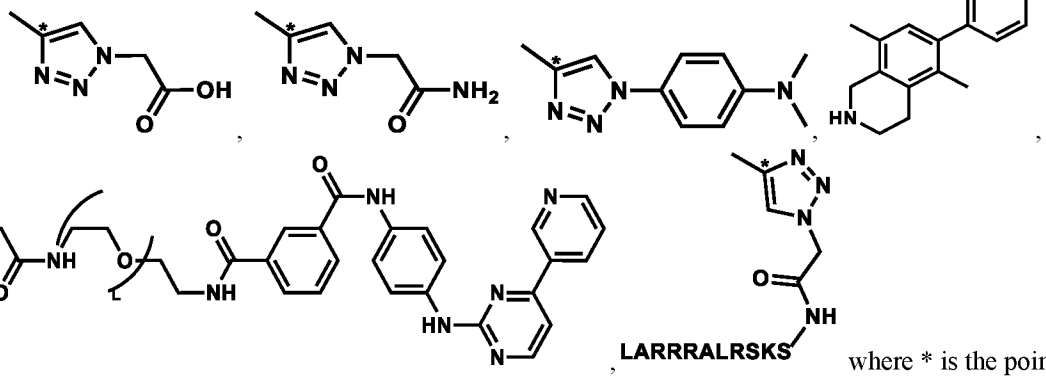


Formula (I-2)

R^2 is independently selected from -C(O)-O-C_{1-3}alkyl, -C(O)-O-C_{2-3}alkenyl, -C(O)-O-C_{2-3} alkynyl, -C(O)-O-aryl, -C(O)-C_{1-3}alkyl, -C(O)-C_{2-3}alkenyl, -C(O)-C_{2-3}alkynyl, -C(O)-aryl, -C(O)-NH_2, -C(O)-O-CH_2-C≡C-phenyl, -C(O)O-CH_2-triazolyl-CH_2-C(O)NH_2; C_{1-3}alkyl, C_{2-3}alkenyl, C_{2-3}alkynyl, carbocycle selected from cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, heterocycle selected from azetidine, oxetane, pyrrolidine, tetrahydrofuran, piperidine, pyran, morpholine, oxazine, dioxane, phenyl, 5-6 membered heteroaryl, halo-C_{1-3}alkyl, phenyl -C_{1-3}alkyl, preferably -C(O)-O-methyl, -C(O)-O-ethyl, -C(O)-O-phenyl, -C(O)-phenyl or -C(O)-O-CH_2-C≡CH, -C(O)-O-CH_2-C≡C-phenyl, C(O)O-CH_2-R^8

R^3 is independently selected from -C(O)-O-C_{1-3}alkyl, -C(O)-O-C_{2-3}alkenyl, -C(O)-O-C_{2-3} alkynyl, -C(O)-O-aryl, -C(O)-C_{1-3}alkyl, -C(O)-C_{2-3}alkenyl, -C(O)-C_{2-3}alkynyl, -C(O)-aryl, -C(O)-NH_2, -C(O)-O-CH_2-C≡C-phenyl, -C(O)O-CH_2-triazolyl-CH_2-C(O)NH_2; preferably -C(O)-O-methyl, -C(O)-O-ethyl, -C(O)-O-phenyl, -C(O)-phenyl or -C(O)-O-CH_2-C≡CH, -C(O)-O-CH_2-C≡C-phenyl, C(O)O-CH_2-R^8

wherein,

R^8 is  where * is the point of attachment and L = 1-3.

9.1. Preferably, the compound according to any of the previous points or pharmaceutically acceptable salts thereof, wherein the compound of formula (I) is a compound of formula (I-1), and wherein one of R^2 and R^3 are as defined herein,
preferably
R^3 is selected from -C(O)-O-C_{1-3}alkyl, -C(O)-O-C_{2-3}alkenyl, -C(O)-O-C_{2-3} alkynyl, -C(O)-O-aryl, -C(O)-C_{1-3}alkyl, -C(O)-C_{2-3}alkenyl, -C(O)-C_{2-3}alkynyl, -C(O)-aryl, -C(O)-NH_2, -C(O)-O-CH_2-C≡C-phenyl; C(O)O-CH_2-R^8
wherein R^8 is as defined in point 1 or 2, or preferably any of points 3 to 8,
preferably -C(O)-O-methyl, -C(O)-O-ethyl, -C(O)-O-phenyl, -C(O)-phenyl or -C(O)-O-CH_2-C≡CH, -C(O)-O-CH_2-C≡C-phenyl,
and R^2 is selected from methyl, cyclohexyl, phenyl, -CH_2-phenyl, -CH_2-CH_2-CH_2Cl, -CH_2-CH=CH_2, 4-Br-phenyl, or together with R^5 may form a -CH_2-C(CH_2)- group, and
R^6 is as defined in any of points 1 to 9, preferably in point 3 or point 4.

9.2. Preferably, the compound according to any of the previous points or pharmaceutically acceptable salts thereof, wherein the compound of formula (I) is a compound of formula (I-2), and wherein one of R^2 and R^3 are as defined herein,
preferably
R^3 is selected from -C(O)-O-C_{1-3}alkyl, -C(O)-O-C_{2-3}alkenyl, -C(O)-O-C_{2-3} alkynyl, -C(O)-O-aryl, -C(O)-C_{1-3}alkyl, -C(O)-C_{2-3}alkenyl, -C(O)-C_{2-3}alkynyl, -C(O)-aryl, -C(O)-NH_2, -C(O)-O-CH_2-C≡C-phenyl, -C(O)O-CH_2-triazolyl-CH_2-$C(O)NH_2$; -C(O)O-CH_2-R^8
wherein R^8 is as defined in point 1 or 2, or preferably any of points 3 to 8,
preferably -C(O)-O-methyl, -C(O)-O-ethyl, -C(O)-O-phenyl, -C(O)-phenyl or -C(O)-O-CH_2-C≡CH, -C(O)-O-CH_2-C≡C-phenyl,
and R^2 is selected from methyl, cyclohexyl, phenyl, -benzyl, -CH_2-CH_2-CH_2Cl, -CH_2-CH=CH_2, 4-Br-phenyl, or together with R^5 may form a -CH_2-$C(CH_2)$- group, and
R_6 is as defined in any of points 1 to 8, preferably in point 2 or point 3.

10. Compound according to any of the previous points or pharmaceutically acceptable salts thereof, wherein

R^6 is 3-7 membered heterocyclyl, C_{6-10} aryl, 5-7 membered heteroaryl, -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{2-4} alkynyl, -C(O)O-C_{6-10} aryl, -C(O)NH_2, -C(O)NHC_{1-4} alkyl, -C(O)NHC_{2-4} alkenyl, -C(O)NHC_{2-4} alkynyl, -C(O)NC_{1-4} $alkyl_2$, -C(O)NC_{2-4} $alkenyl_2$, -C(O)NC_{2-4} $alkynyl_2$, -C(O)NH-5-10 membered heteroaryl, -C(O)O-C_{1-4} alkyl-C≡CR^7, -C(O)O-C_{1-4}-alkyl-R^8

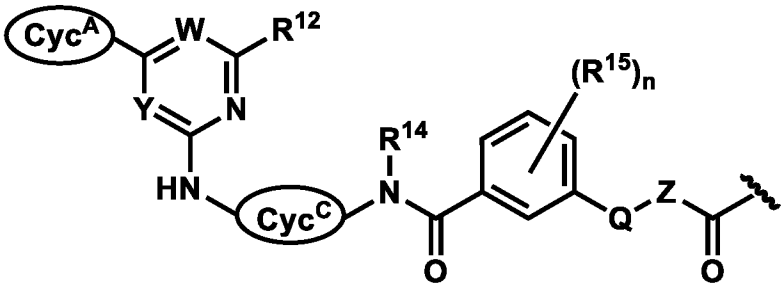
R^7 is H, C_{6-10} aryl, 5-7 membered heteroaryl, vinyl, -$[(CH_2)_2O]_n$-C(O)OH, -$[(CH_2)_2O]_n$-C(O)OC_{1-4} alkyl, -$[(CH_2)_2O]_n$-$C(O)NH_2$, -$[(CH_2)_2O]_n$-C(O)NHC_{1-4} alkyl, -$[(CH_2)_2O]_n$-C(O)NC_{1-4} $alkyl_2$-$[(CH_2)_2O]_n$-NH_2, -$[(CH_2)_2O]_n$-NHC_{1-4} alkyl, -$[(CH_2)_2O]_n$-NC_{1-4} $alkyl_2$, -C(O)O-$[(CH_2)_2O]_n$- C_{6-10} aryl, -C(O)O-$[(CH_2)_2O]_n$-5-7 membered heteroaryl, -C(O)NH$(CH_2)_n$NHC(O) C_{6-10} aryl, -C(O)NH$(CH_2)_n$NHC(O)-5-7 membered heteroaryl, preferably H, C_{6-10} aryl, 5-7 membered heteroaryl

R^8 is 5-6-membered heterocyclyl, the heterocyclyl is optionally substituted with one or more substituent independently selected from C_{1-4} alkyl, C_{2-4} alkenyl, C_{2-4} alkynyl, C_{3-6} carbocyclyl, 3-6 membered heterocyclyl, C_{6-8} aryl, 5-10 membered heteroaryl, the aryl and heteroaryl are optionally substituted with one or more substituent independently selected from C_{1-4} alkyl, halogen, -NH_2, -NHC_{1-4} alkyl, -$N(C_{1-4}$ $alkyl)_2$;
halo-C_{1-4} alkyl, aryl-C_{1-4} alkyl, -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{6-10} aryl, -C(O)-C_{1-4} alkyl, -C(O)-C_{2-4} alkenyl, -C(O)-C_{2-4} alkynyl, -C(O)-C_{6-10} aryl, -C(O)NH_2, -C(O)NHC_{1-4} alkyl, -C(O)NHC_{2-4} alkenyl, -C(O)NHC_{2-4} alkynyl, -C(O)NC_{1-4} $alkyl_2$, -C(O)NC_{2-4} $alkenyl_2$, -C(O)NC_{2-4} $alkynyl_2$, -CN, -SO_3H, -SO_3C_{1-4} alkyl, -CF_3, -C_{1-4} alkyl-C(O)O-C_{1-4} alkyl, -C_{1-4} alkyl-C(O)OH, -C_{1-4} alkyl-C(O)O-C_{2-4}alkenyl, -C_{1-4}alkyl-C(O)NH_2, -C_{1-4} alkyl-C(O)NH_2, -C_{1-4} alkyl-C(O)NHC_{1-4} alkyl, -C_{1-4} alkyl-C(O)NC_{1-4} $alkyl_2$, -$[(CH_2)_2O]_n$-C(O)OH, -$[(CH_2)_2O]_n$-C(O)OC_{1-4} alkyl, -$[(CH_2)_2O]_n$-NH_2, -$[(CH_2)_2O]_n$-NHC_{1-4} alkyl, -$[(CH_2)_2O]_n$-NC_{1-4} $alkyl_2$, -CH_2-C(O)NH$(CH_2)_n$NHC(O)-C_{6-10} aryl, -CH_2-C(O)O$(CH_2)_n$NHC(O)-C_{6-10} aryl, -CH_2-C(O)NH$(CH_2)_n$OC(O)-C_{6-10} aryl, -CH_2-C(O)O$(CH_2)_n$OC(O)-C_{6-10} aryl, C(O)NH$(CH_2)_n$NHC(O)-5-7 membered heteroaryl wherein
n = 1-6.

11. Compound according to any of the previous points or pharmaceutically acceptable salts thereof, wherein

R^6 is -C(O)O-methyl, -C(O)O-ethyl, -C(O)O-*tert*butyl, -C(O)O-CH_2-phenyl, -C(O)NH-*tert*butyl, -C(O)NH-phenyl, -C(O)NH-CH_2-phenyl, -C(O)NH-$C(Me)_2$-CH_2-OH, -C(O)NH-CH_2-$C(Me)_2$-OH, preferably -C(O)O-*tert*butyl, -C(O)Obenzyl, 5-methyloxazol-2-yl, quinolin-8-yl.

12. Compound according to any of points 1-9 or pharmaceutically acceptable salts thereof, wherein



R^6 is

wherein

Cyc^A is an optionally substituted aryl or heteroaryl ring, preferably an optionally substituted phenyl or pyridine ring, more preferably an optionally substituted 3-pyridinyl ring; even more preferably an unsubstituted 3-pyridinyl ring;

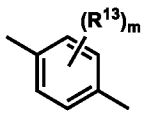
Y is N or CR^{10}, preferably N

W is CR^{11} or N, preferably CR^{11}

R^{10} is H or C_{1-6} alkyl, preferably H

R^{11} is H or C_{1-6} alkyl, preferably H

R^{12} is H, halogen, C_{1-6} alkyl, preferably H or Cl, more preferably H

Cyc^C is phenyl optionally substituted with R^{13} group(s) or a phenyl isostere, preferably  or 

R^{13} is halogen, C_{1-6} alkyl, halo-C_{1-6} alkyl, preferably Cl, methyl or trifluoromethyl, more preferably methyl

m is an integer of 0 to 4, preferably 0 or 1

R^{14} is H, C_{1-6} alkyl or N-protecting group, preferably H, methyl, ethyl, or an N-protecting group, more preferably H

R^{15} is halogen, C_{1-6} alkyl, halo-C_{1-6} alkyl, preferably C_{1-3} alkyl, preferably halogen, methyl, ethyl, or trifluoromethyl, preferably H or methyl,

n is an integer of 0 to 4, preferably 0, 1 or 2, more preferably 0 or 1

Q is direct bond or $-[-CHR^{16}-]_o-$

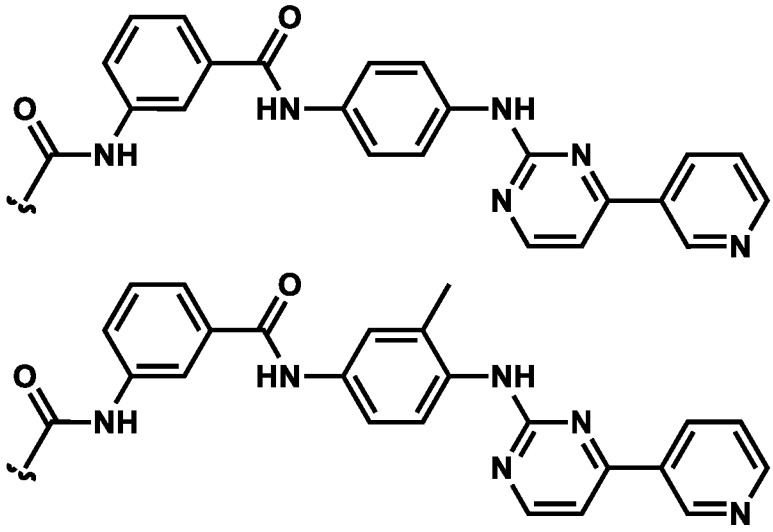
R^{16} is H or C_{1-6} alkyl, preferably H

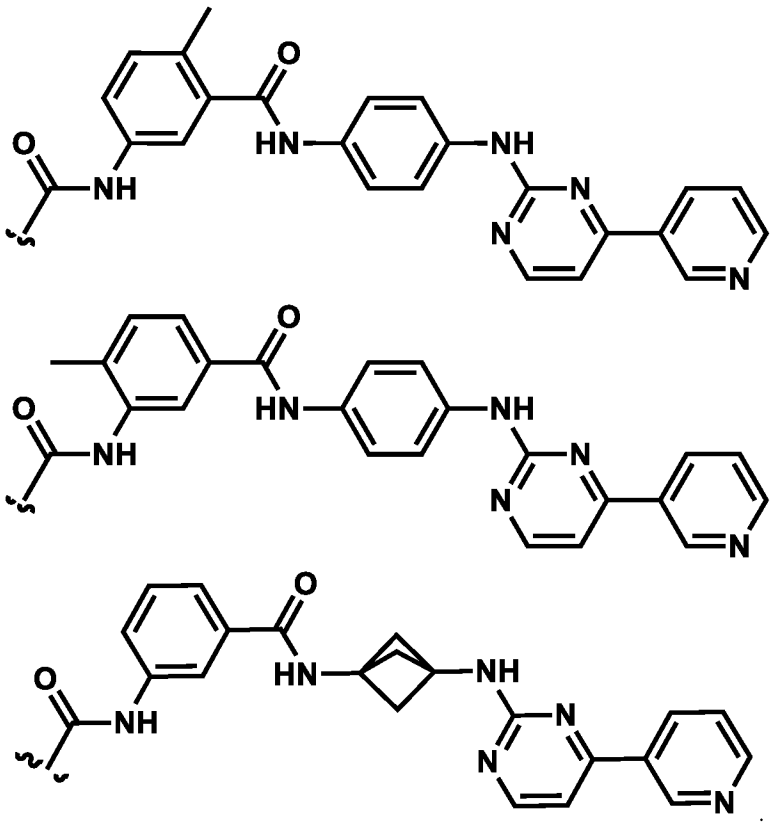
o is an integer of 1 or 2, preferably 1

Z is NH or O

13. Compound according to point 11 or pharmaceutically acceptable salts thereof, wherein

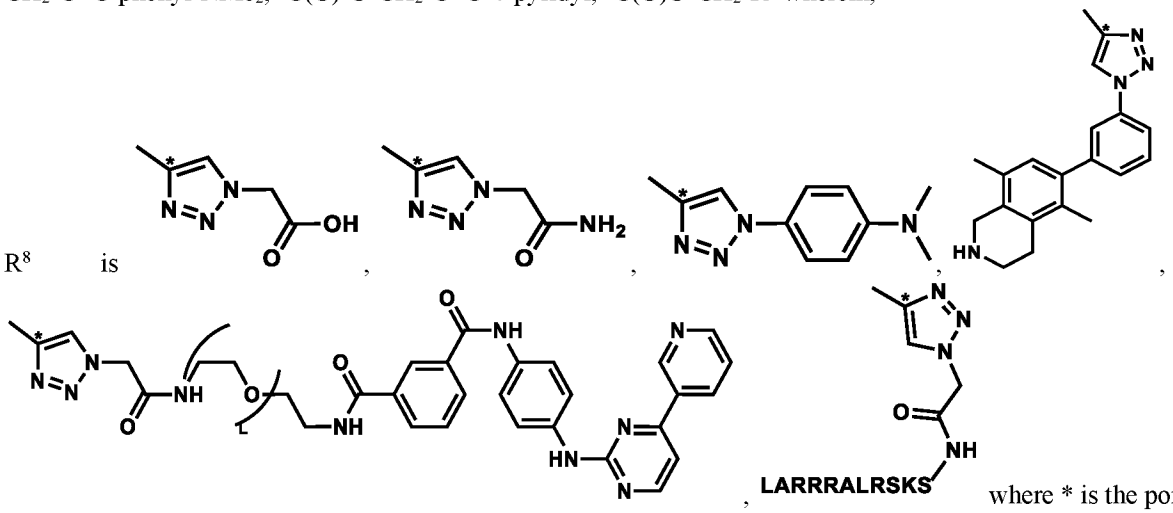
R^6 is selected from





14. Compound according to any of points 1-10 or pharmaceutically acceptable salts thereof, wherein

R^3 is C(O)-O-C_{1-4}alkyl, C(O)-O-C_{2-3}alkynyl, C(O)-aryl; preferably methyl, C(O)-O-methyl, C(O)-O-ethyl, C(O)-phenyl or -C(O)-O-CH_2-C≡CH, -C(O)-O-$(CH_2)_2$-C≡CH, -C(O)-O-$(CH_2)_3$-C≡CH, -C(O)-O-$(CH_2)_4$-C≡CH, -C(O)-O-CH_2-C≡C-phenyl-NMe_2, -C(O)-O-CH_2-C≡C-4-pyridyl, -C(O)O-CH_2-R^8 wherein,

R^8 is 

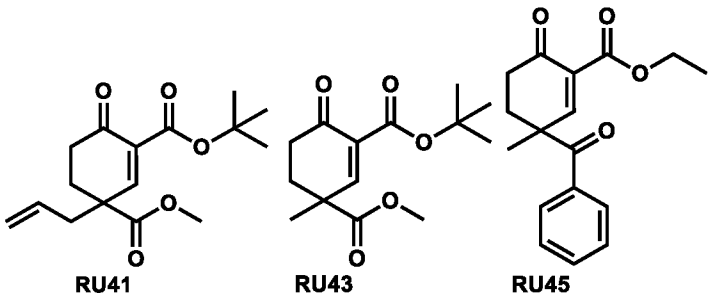
where * is the point of attachment and
L = 1-2

R^2 is C_{1-3}alkyl, C_{2-3}alkenyl, C_{2-3}alkynyl, cyclohexyl, halo-C_{1-3}alkyl, phenyl -C_{1-3}alkyl, preferably methyl, cyclohexyl, phenyl, benzyl, halo-C_{1-3}alkyl, -CH_2-CH_2-CH_2Cl, -CH_2-CH=CH_2,

or R^2 together with R^5 may form -CH_2-C(CH_2)-,

R^6 is -C(O)O^{t}Bu, -C(O)Obenzyl.

15.1. Compound according to point 1 or pharmaceutically acceptable salts thereof, wherein the compound is selected from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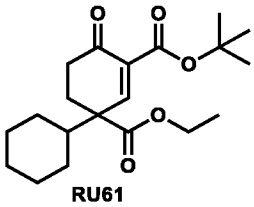
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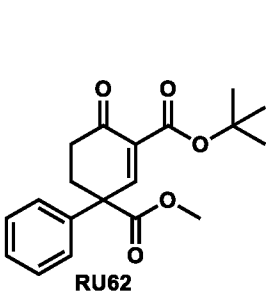
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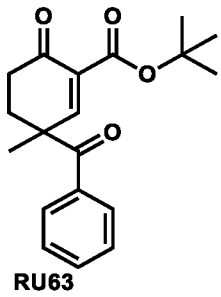
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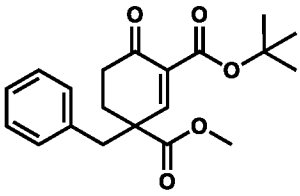
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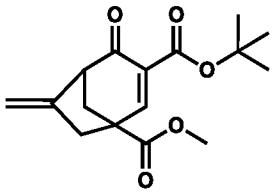
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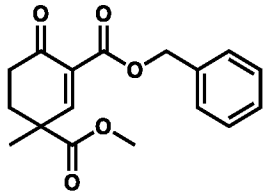
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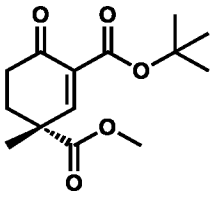
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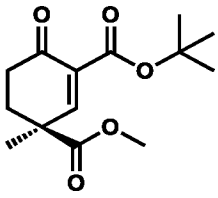
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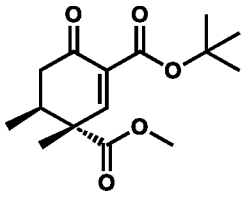
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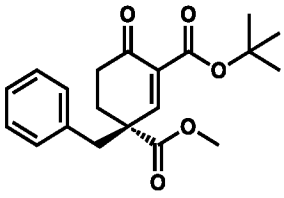
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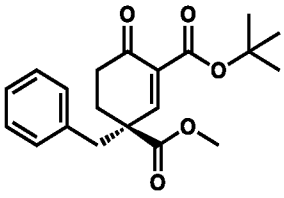
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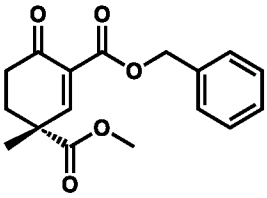
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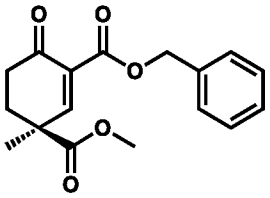
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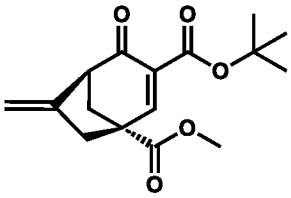
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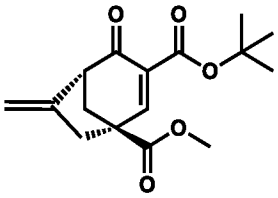
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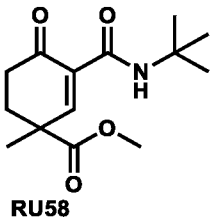
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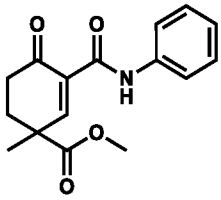
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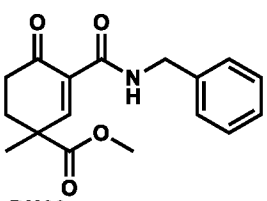
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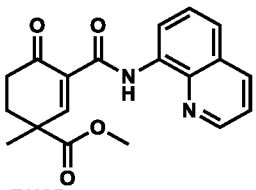
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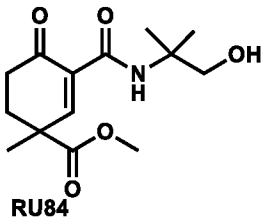
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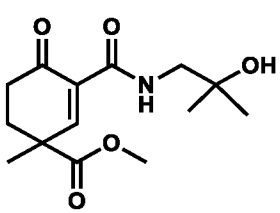
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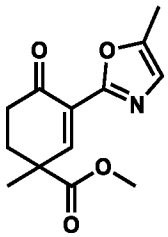
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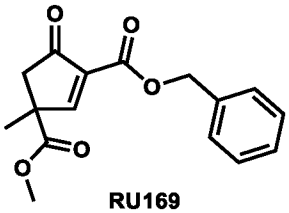
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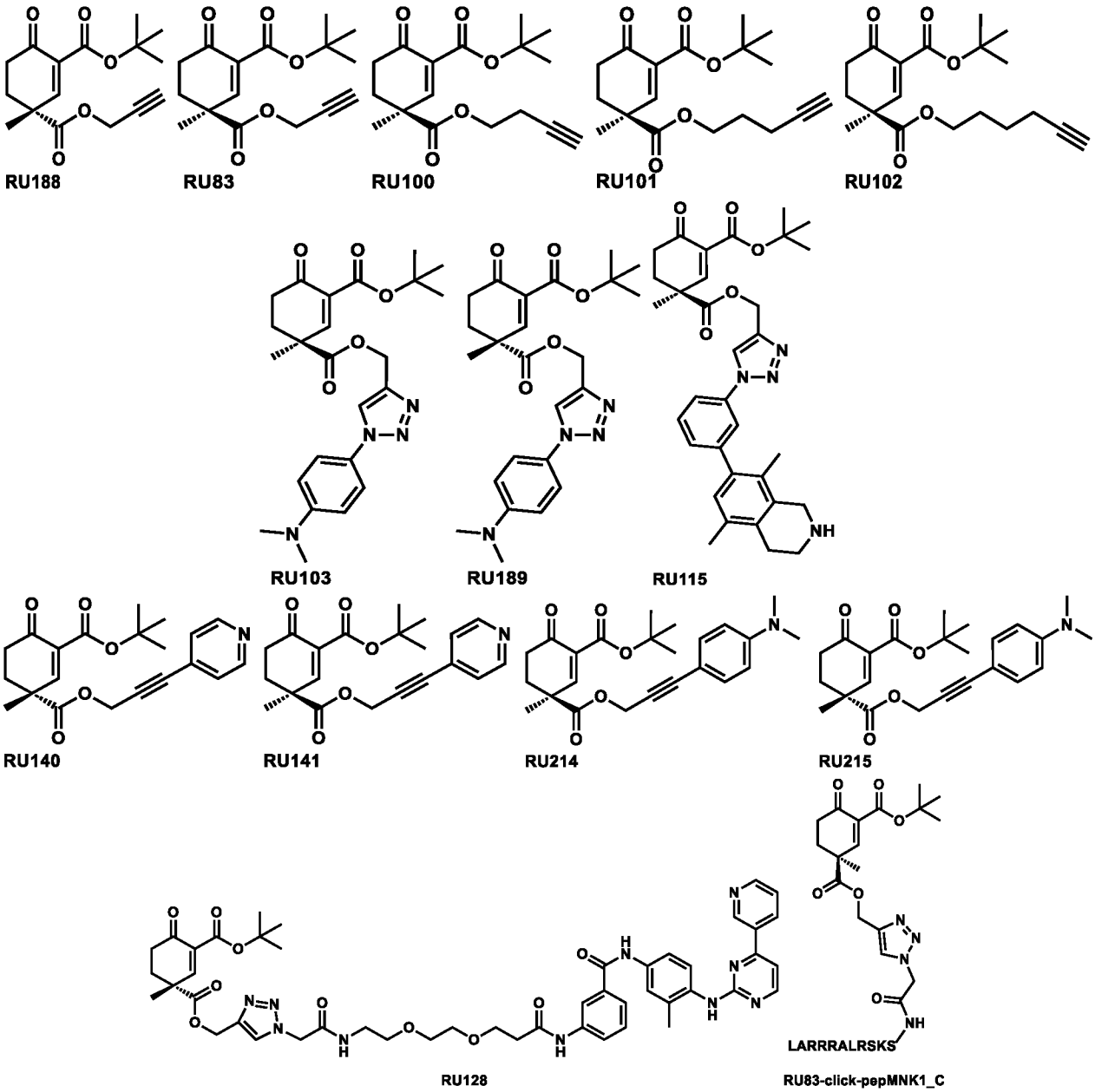
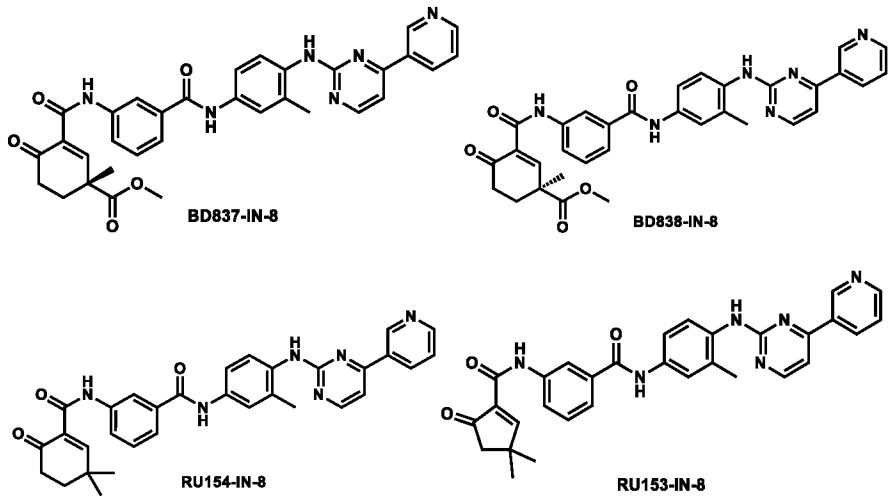
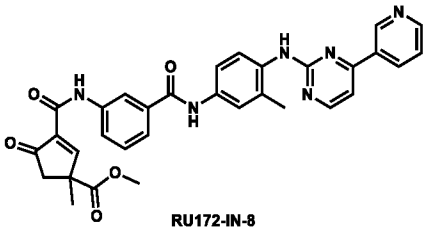


15.2. Compound according to point 1 or pharmaceutically acceptable salts thereof, wherein the compound is selected from

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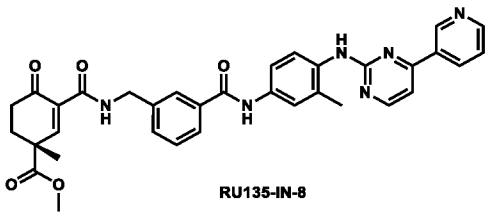
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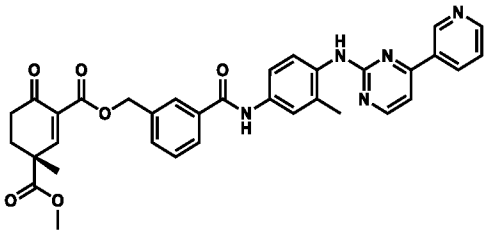
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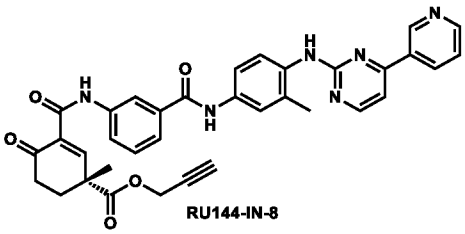
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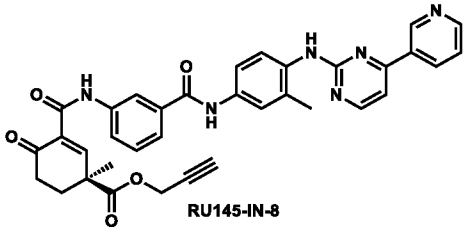
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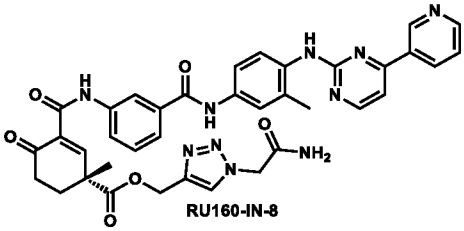
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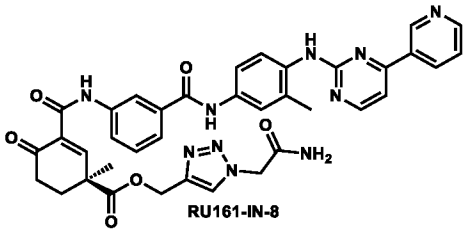
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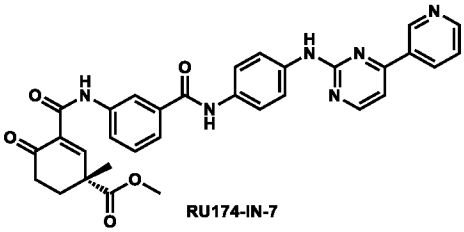
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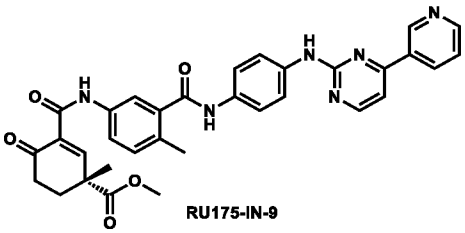
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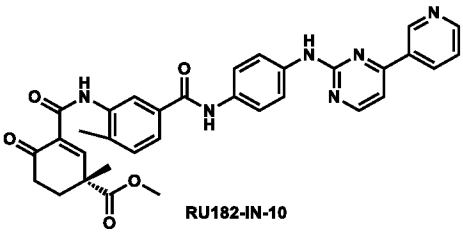
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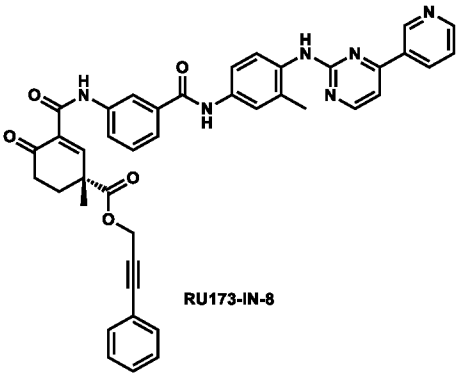
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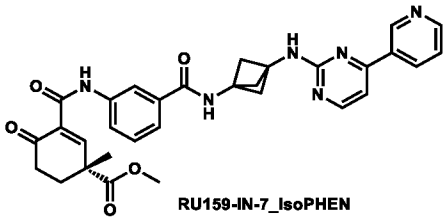
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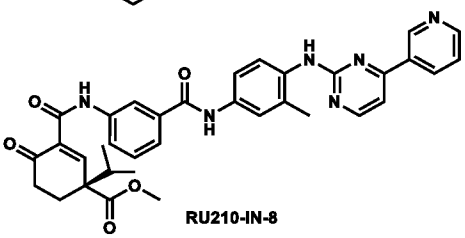
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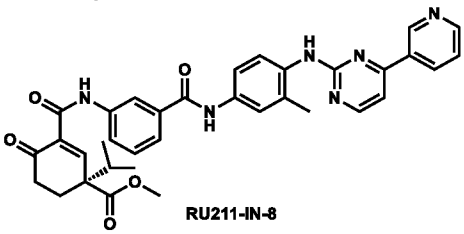
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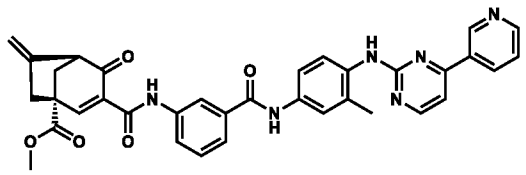

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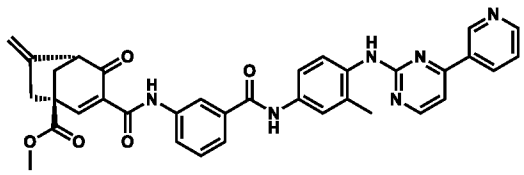
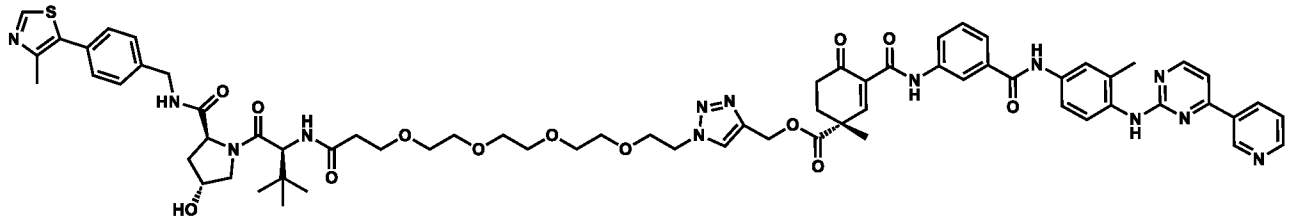


RU213-IN-8

15.3 Compound according to point 1 or pharmaceutically acceptable salts thereof, wherein the compound is selected from



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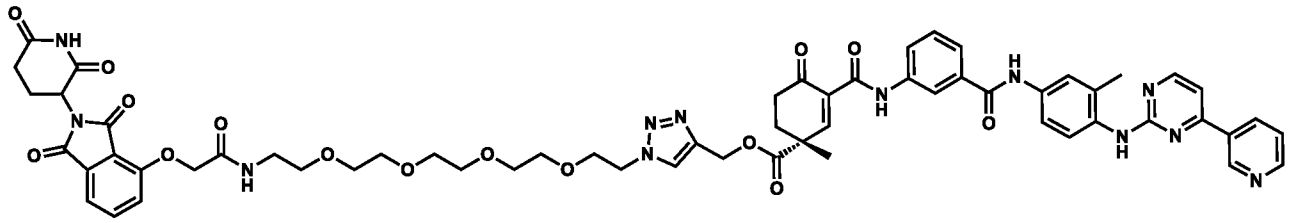

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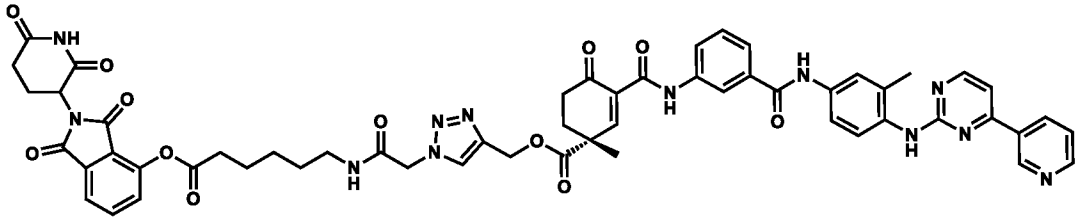
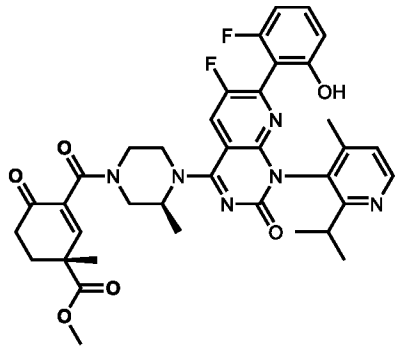


RU232.

15.4. Compound according to point 1 or pharmaceutically acceptable salts thereof, that is



RU230

16. The **compound** of any one of points 1 to 15 which binds to a protein as a Michael-acceptor type protein binding compound or molecule.
Preferably said binding is covalent and reversible.
Said binding is detectable by any assay suitable to detect protein binding of organic molecules, for example surface plasmon resonance (SPR), isothermal calorimetry (ITC), fluorescence polarization (FP) or NanoBiT assay.
The Michael-acceptor compound of any of points 1 to 15 is capable of binding in particular to a Cys and/or a His of a protein, as a nucleophile.
In particular the protein binding Michael-acceptor compound binds to a Cys and/or a His residue as a nucleophile.

16.1 In particular the protein binding Michael-acceptor compound is a Michael-acceptor type protein inhibitor, wherein activity of a protein is inhibited by binding of the compound of the invention to said protein. In particular binding is targeted to a site of said protein which is essential to protein activity, whereas the warhead moiety of the compound of the invention covalently and reversibly binds to a nucleophile amino acid residue. In particular the nucleophile amino acid is a Cys and/or a His residue.

In an embodiment the site essential to protein activity is preferably a binding surface.
Preferably the binding surface is a part of the protein surface to which another protein, e.g. a binding partner, preferably a protein substrate/activator/deactivator/scaffold protein or protein domain is capable of binding which is essential to the activity of said protein to which the Michael-acceptor compound of the invention binds.

In a further embodiment the site essential to protein activity is preferably an active site region, wherein a part of the compound of the invention interferes with the active site of the protein.
The active site region comprises an appropriate nucleophile residue, preferably a Cys and/or a His residueto which the warhead moiety of the Michael-acceptor compound of the invention binds; as well as the active site. Preferably the nucleophile residue and the active site of said protein is in a sufficient proximity whereby the Michael-acceptor compound, once bound, interferes with the activity of said protein.
Said inhibition is detectable by any assay suitable to detect protein activity or inhibition of activity of said protein, for example biochemical enzymatic assays in vitro or cell-based assays capable of detecting protein levels or activity in cells (e.g. by western blotting using specific antibodies).
In an embodiment the Cys is a conservative Cys of the protein molecule.
In an embodiment the His is a conservative His of the protein molecule.
In an embodiment the Cys is not a conservative Cys of the protein molecule.
In an embodiment the His is not a conservative His of the protein molecule.

16.2 In a further particular embodiment the protein binding Michael-acceptor compound is a (part of a) bifunctional molecule, comprising a Michael-acceptor type protein binding moiety and a further protein binding moiety capable of binding to a further protein and preferably a linker between the Michael-acceptor type protein binding molecule or moiety and the further protein binding moiety.
In an alternative wording, the protein binding Michael-acceptor compound is a moiety of a bifunctional molecule, comprising the Michael-acceptor type protein binding moiety.
In an embodiment the bifunctional molecule is a homobifunctional molecule wherein the protein binding function of said bifunctional molecule is identical, i.e. bind the same protein, i.e. the protein and said further protein are identical.
In a preferred embodiment the bifunctional molecule is a heterobifunctional molecule wherein the protein binding functions of said bifunctional molecule are different, preferably bind different proteins, i.e. said protein and said further protein are different.
In an embodiment the further protein binding moiety capable of binding to a further protein is a Michael-acceptor type protein binding moiety as defined herein.

In a preferred embodiment the further protein is a protein the function of which is to be utilized in connection with said protein.
In a particular embodiment the further protein is a ligase protein. In a particularly preferred embodiment the further protein is a ubiquitin ligase capable of ubiquitination of said protein as a target protein. Preferably the compound of the invention is a part of a proteolysis targeting chimera (PROTAC). Preferably the invention provides a proteolysis targeting chimera comprising the compound of any one of points 1 to 16.1.

17. The **use** (preferably *ex vivo* use) **of a compound** of any one of points 1 to 16 as a Michael-acceptor type **protein binding molecule**.
Said binding is covalent and reversible.
Said binding is detectable by any assay suitable to detect protein binding as defined in point 16.
The Michael-acceptor compound of any of points 1 to 16 is capable of binding in particular to a Cys and/or a His of a protein, as a nucleophile.
In particular the protein binding Michael-acceptor compound binds to a Cys and/or a His residue as a nucleophile.

In a preferred embodiment the invention relates to the use, preferably *ex vivo* use of a Michael-acceptor compound of any one of points 1 to 16 as a protein activity modulator, wherein the compound binds to a Cys and/or His residue of said protein with a reversible covalent bond.
In particular, binding of the Michael-acceptor compound of the invention modifies protein activity, whereas the warhead moiety of the compound of the invention covalently and reversibly binds to a nucleophile amino acid residue. In particular the nucleophile amino acid is a Cys and/or a His residue.
In an embodiment the site essential to protein activity is a binding surface.

Preferably the binding surface is a part of the protein surface to which another protein, e.g. a binding partner can bind.
In an embodiment the binding partner may be a protein inhibitor whereby the Michael-acceptor compound, by blocking the binding of the inhibitor, serves as an activator of said protein. In an other embodiment the binding partner is a protein agonist of said protein and the Michael-acceptor compound, by blocking the binding of the agonist, serves as an inhibitor of said protein.
Preferably the binding surface is as defined in the previous point(s) or as defined herein.
In an embodiment the Cys is a conservative Cys of the protein to which the binding partner can bind.
In an embodiment the His is a conservative His of the protein to which the binding partner can bind.
In an embodiment the Cys is not a conservative Cys of the protein to which the binding partner can bind.
In an embodiment the His is not a conservative His of the protein to which the binding partner can bind.

17.1 Preferably, the invention relates to the **use** (preferably *ex vivo* use) **of a compound** of any one of points 1 to 16 as a Michael-acceptor type **protein inhibitor**.
In particular, binding of the Michael-acceptor type protein inhibitor of the invention is targeted to a site of said protein which is essential to protein activity, whereas the warhead moiety of the compound of the invention covalently and reversibly binds to a nucleophile amino acid residue. In particular the nucleophile amino acid is a Cys and/or a His residue.
In an embodiment the site essential to protein activity is preferably a binding surface.
Preferably the binding surface is as defined in the previous point(s) or as defined herein.

In a further embodiment the site essential to protein activity is preferably an active site region.
Preferably the active site region is as defined in the previous point(s) or as defined herein.
Said inhibition is detectable by any assay suitable to detect protein activity or inhibition of activity of said protein, for example biochemical enzymatic assays in vitro (e.g. PhALC assay) or cell-based assays capable of detecting protein levels or activity in cells (e.g. by western blotting using specific antibodies).
In a particular embodiment the compound binds to a conservative Cys of an ATPase domain in said protein.
In a particular embodiment the compound binds to a conservative His of an ATPase domain in said protein.
In a particular embodiment the compound binds to a conservative Cys of an ATPase active site region in said protein, preferably in proximity of an ATP binding site.
In a particular embodiment the compound binds to a conservative His of an ATPase active site region in said protein, preferably in proximity of an ATP binding site.
In an alternative embodiment the Cys is not a conservative Cys.
In an alternative embodiment the His is not a conservative His.

Preferably said protein is a kinase and the compound of the invention is used as a kinase inhibitor.
Preferably the compound is as defined in point 16.

17.2 In a further particular embodiment the protein binding Michael-acceptor compound is **used as a moiety of a bifunctional molecule**, as defined in point 16.1.
Preferably the bifunctional molecule comprises the Michael-acceptor type protein binding moiety and a further protein binding moiety capable of binding to a further protein and preferably a linker between the Michael-acceptor type protein binding moiety and the further protein binding moiety.
In a particular embodiment the further protein is a ligase protein. In a particularly preferred embodiment the further protein is a ubiquitin ligase capable of ubiquitination of said protein as a target protein.
In a highly preferred embodiment the compound of the invention is used as a moiety, which is a part of a proteolysis targeting chimera (**PROTAC**).

In a highly preferred embodiment the one or more of the disclaimers defined in point 1.1 do not apply in the use of said compound as a kinase inhibitor.
In a particular embodiment the following disclaimer does not apply:
the disclaimer defined in point 1.1.1,
the disclaimer defined in point 1.1.2,
the disclaimer defined in point 1.1.3,
the disclaimer defined in point 1.1.4,
the disclaimer defined in point 1.1.5,
the disclaimer defined in point 1.1.6,
the disclaimer defined in point 1.1.7.

18. The *ex vivo* use of a compound of any one of points 1 to 16 as kinase inhibitor.
The *ex vivo* use of point 17.1 wherein said compound of any one of points 1 to 16 is used as a kinase inhibitor.

Preferably the kinase inhibitor is an inhibitor of a kinase of the family of mitogen-activated protein kinases (MAP kinases or MAPK).

Preferably the kinase inhibitor is an inhibitor of a kinase of the MAPK subfamily of extracellular regulated kinases 1 (ERK1).
Preferably the kinase inhibitor is an inhibitor of a kinase of the MAPK subfamily of extracellular regulated kinases 2 (ERK2).
Preferably the kinase inhibitor is an inhibitor of a kinase of the MAPK subfamily of c-Jun N-terminal kinases (JNK).
Preferably the kinase inhibitor is an inhibitor of a kinase of the MAPK subfamily of p38 kinases.
Preferably the kinase inhibitor is an inhibitor of a kinase of the MAPK subfamily of extracellular regulated kinases 5 (ERK5).
In a particular embodiment the kinase inhibitor is selected from the following kinases:
ERK1/2, JNK, p38.
In a more preferred embodiment the kinase is selected from the following kinases:
ERK2, JNK1, JNK2, JNK3, p38alpha.

In a highly preferred embodiment the one or more of the disclaimers defined in point 1.1 do not apply in the use of said compound as a kinase inhibitor.
In a particular embodiment the following disclaimer does not apply:
the disclaimer defined in point 1.1.1,
the disclaimer defined in point 1.1.2,
the disclaimer defined in point 1.1.3,
the disclaimer defined in point 1.1.4,
the disclaimer defined in point 1.1.5,
the disclaimer defined in point 1.1.6,
the disclaimer defined in point 1.1.7.

In a preferred embodiment the compound binds to the kinase as measured by fluorescence polarization (FP), in particular to a Cys and/or His of said kinase.
In a particular embodiment the Cys is a conservative Cys of said kinase protein, preferably of said kinase family or kinase subfamily.
In a particular embodiment the His is a conservative His of said kinase protein, preferably of said family or kinase subfamily.
In an alternative embodiment the Cys is not a conservative Cys.
In an alternative embodiment the His is not a conservative His.

In a preferred embodiment the compound is measured as an inhibitor by any of the following assays:
Z'LYTE Kinase Assay, ADP-Glo Assay, NanoBRET target engagement assay, ^{33}PanQinase kinase assay

Highly preferably the inhibiting effect of the compound of the invention can be measured by any of the following assays:
Phosphorylation-Assisted Luciferase Complementation (PhALC) assay (Poti et al, 2023) or NanoBiT luciferase fragment-complementation assay (Dixon et al, 2016).

In a highly preferred embodiment the compound is a compound as defined in
point 2, or any of points 2 to 9, or preferably point 9,
in particular point 9.1 or
in particular point 9.2 or
preferably any of points 10 to 15,
preferably in point 16, e.g. 16.1.

19. The *ex vivo* use of a compound of any one of points 1 to 16 as a kinase inhibitor as defined in point 18, wherein said compound binds to the MAPK D-groove.
Preferably the compound binds to a conservative Cys and/or a His residue of said protein with a reversible covalent bond.
Preferably the compound is as defined in point 16 or 18.

Preferably the compound binds to a conservative Cys residue of said protein with a reversible covalent bond.
Preferably the compound is RU60, RU67, RU68, RU75, RU76, RU128, RU81, RU169, RU188, RU83, RU100, RU101, RU102, RU103, RU189, RU115, RU140, RU141, RU214, RU215

Preferably the compound binds to a conservative His residue of said protein with a reversible covalent bond.
Preferably the compound is RU77.

Preferably the compound binds to either to a conservative Cys or a His residue of said protein with a reversible covalent bond.

Preferably the compound is RU187.

20. The compound of any of claims 1 to 16 **for use in the treatment of a disease in a subject**.
The compound of any of claims 1 to 16 **for use in modulating a** drug target protein associated with a disease.
Preferably the Michael-acceptor compound of the invention is used as an inhibitor of a drug target protein associated with said disease.
Preferably the Michael-acceptor compound of the invention is used as a kinase inhibitor.
Preferably said disease is selected from the group consisting of an autoimmune disease, an inflammatory disease, a cardiovascular disease, a neurological disease or a disease of the nervous system, or a neoplasm or a cancer or malignancy, preferably a disease wherein kinase inhibition is required.

In a particular embodiment treatment involves prevention or avoiding or delaying the onset of a disease.

In a highly preferred embodiment the one or more of the disclaimers defined in point 1.1 do not apply in the use of said compound as a kinase inhibitor.
In a particular embodiment the following disclaimer does not apply:
the disclaimer defined in point 1.1.1,
the disclaimer defined in point 1.1.2,
the disclaimer defined in point 1.1.3,
the disclaimer defined in point 1.1.4,
the disclaimer defined in point 1.1.5,
the disclaimer defined in point 1.1.6,
the disclaimer defined in point 1.1.7.

In a preferred embodiment the compound in a kinase inhibitor as defined in any of claims 1 to 15 or in any of claims 16 to 19.

21. The compound for use according to point 20 for use in the treatment of a subject in need of inhibition of a kinase activated pathway.
Preferably the kinase activated pathway is a MAPK kinase activated pathway, wherein preferably the disease is selected from the group consisting of an autoimmune disease, an inflammatory disease, a cardiovascular disease, a neurological disease or a disease of the nervous system, or a neoplasm or a cancer or malignancy,
preferably an inflammatory disease, a neurological disease or a neoplasm or any variant thereof as defined herein.
Preferably the kinase activated pathway is selected from
an ERK1 kinase activated pathway,
an ERK2 kinase activated pathway,
an ERK5 kinase activated pathway,
a JNK1 kinase activated pathway,
a JNK2 kinase activated pathway,
a JNK3 kinase activated pathway,
a p38alpha kinase activated pathway,
a p38beta kinase activated pathway,
a p38gamma kinase activated pathway,
p38delta kinase activated pathway.
Preferably the kinase activated pathway is selected from
an ERK1 kinase activated pathway,
an ERK2 kinase activated pathway,
a JNK kinase activated pathway,
a p38 kinase activated pathway or
an ERK5 kinase activated pathway.
In a particular embodiment the kinase activated pathway is an ERK1/2, JNK and/or p38 kinase activated pathway, more preferably ERK2, JNK1, JNK2, JNK3, p38alpha kinase activated pathway.
Preferably the kinase activated pathway is an ERK2 kinase activated pathway, wherein preferably the disease is selected from the group consisting of an autoimmune disease, an inflammatory disease, a cardiovascular disease, a neurological disease or a disease of the nervous system, or a neoplasm or a cancer or malignancy,
preferably an inflammatory disease, a neurological disease or a neoplasm or any variant thereof as defined herein.
Preferably the kinase activated pathway is a JNK1 kinase activated pathway, wherein preferably the disease is selected from the group consisting of an autoimmune disease, an inflammatory disease, a cardiovascular disease, a neurological disease or a disease of the nervous system, or a neoplasm or a cancer or malignancy,
preferably an inflammatory disease, a neurological disease or a neoplasm or any variant thereof as defined herein.
Preferably the kinase activated pathway is a p38 kinase activated pathway, wherein preferably the disease is selected from the group consisting of an autoimmune disease, an inflammatory disease, a cardiovascular disease, a neurological disease or a disease of the nervous system, or a neoplasm or a cancer or malignancy,

preferably an inflammatory disease, a neurological disease or a neoplasm or any variant thereof as defined herein. In a particular embodiment the kinase activated pathway is an ERK1/2, JNK and/or p38 activated pathway, more preferably ERK2, JNK1, JNK2, JNK3, p38alfa activated pathway, wherein preferably the disease is selected from the group consisting of an autoimmune disease, an inflammatory disease, a cardiovascular disease, a neurological disease or a disease of the nervous system, or a neoplasm or a cancer or malignancy,
preferably an inflammatory disease, a neurological disease or a neoplasm or any variant thereof as defined herein.

22. The compound for use according to point 21 wherein said disease is selected from the group consisting of the following diseases:

type 2 diabetes mellitus and obesity associated with insulin resistance

a neoplasm or a cancer or malignancy,
preferably a tumor,
in particular a solid tumor, for example a breast, lung or colon tumor,

a neurological disease or a disease of the nervous system,
preferably selected from the group consisting of
neurological injuries, like acute brain injury, such as ischemic stroke (IS), intracerebral hemorrhage (ICH), subarachnoid hemorrhage (SAH), traumatic brain injury (TBI), spinal cord injury (SCI), epilepsy, etc;
neurodegenerative diseases, such as Huntington's disease (HD), Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), etc;
neurodevelopment diseases, such as autism spectrum disorder (ASD) and cerebral palsy, as well as others;
infectious neurological diseases, such as meningitis and encephalitis; and
neurological tumors, such as neuroblastoma (NB), glioblastoma (GBM), glioma, etc.

an inflammatory disease,
preferably atopic dermatitis, arthritis, e.g. psoriatic arthritis, rheumatoid arthritis, Crohn disease, ulcerative colitis, atherosclerosis, multiple sclerosis (MS), chronic obstructive pulmonary disease (COPD) and asthma.

In particular the disease is a disease related to impaired cell cycle, in particular selected from neoplasm and a neurological disease which is a cell cycle impairment disease or an aberrant cell cycle disease.
Preferably the kinase activated pathway is a JNK kinase activated pathway and the disease is selected from stomach cancer, oral squamous carcinoma, lung adenocarcinoma, cholangiocarcinoma, colon carcinoma, pancreatic cancer, glioblastoma, colon cancer, leukemia.
Preferably the kinase activated pathway is a p38 kinase activated pathway and the disease is selected from multiple myeloma, leukemia, oral epidermoid carcinoma, cervical cancer, melanoma, non-small cell lung cancer, ovarian cancer, glioma, myeloma, breast cancer, lung adenocarcinoma, colorectal, sarcoma, NSCLC, renal cancer, pancreatic cancer, and ovarian cancer.
Preferably the kinase activated pathway is an ERK1/2 kinase activated pathway and the disease is colorectal cancer.
Preferably the kinase activated pathway is an ERK5 kinase activated pathway and the disease is selected from lung cancer, cervical cancer, acute myeloid leukemia, pancreatic cancer, hepatocellular carcinoma, colon cancer.

23. The compound for use according to any of points 20 to 22, wherein the kinase inhibitor is an inhibitor of a kinase of the family of mitogen-activated protein kinases (MAP kinases or MAPK), preferably the kinase is selected from extracellular regulated kinases 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), p38 kinase, and extracellular regulated kinase 5 (ERK5), preferably from ERK2, JNK1, p38.
Preferably, the compound for use according to the invention binds to the MAPK D-groove.
Preferably the compound binds to a Cys or His residue of said protein which is conservative in MAP kinases, preferably in the MAPK D-groove, and binds with a reversible covalent bond.
Preferably the compound is as defined in point 16, 17 or 18.

24. In a highly preferred embodiment the invention relates to a pharmaceutical composition comprising the compound as defined in
point 2, or any of points 2 to 9, or preferably point 9,
in particular point 9.1 or
in particular point 9.2 or
preferably any of points 10 to 15,
and a pharmaceutically acceptable excipient or carrier.

25. The invention also relates to a method of treatment wherein a pharmaceutical composition as defined in point 24 is administered to a subject in need thereof.
Preferably the compound is administered in sufficient dose by any appropriate means preferably as defined herein.

Preferably the subject is suffering in or jeopardized by a disease as defined in any of points 20 to 23.

Brief description of the drawings

It is noted that some of the structures shown in the Figures fall outside the scope of the appended claims. These have, however, been retained for reference only.

Figure 1 shows the location of the MAPK D-groove cysteine (ERK2: Cys161, p38α: Cys162, and JNK1: Cys163). The panels show the crystallographic models of ERK2, p38α, and JNK1 bound to D-peptides (shown in black). The sidechain atoms of the cysteines are shown with spheres. The panels below show examples of the competitive fluorescence polarization-based protein-peptide assay for ERK2, p38α, and JNK1. Panels on left show the direct titration curve using a fluorescently labelled peptide known to bind to the MAPK D-groove: a fixed amount of the labelled peptide was titrated with increasing amounts of human MAPKs (ERK2, p38α, JNK1; wt: wild-type) and fluorescence polarization (FP) in arbitrary units was monitored. This gives the dissociation constant (K_d) for the binding of the labelled peptide with the respective MAPK. Panels on the right show the competitive binding curves with two compounds. (The MAPK concentration was chosen to start out with ~50-90% complex formation with the labelled peptide; no competitor added; FB: fraction bound: ~0.5-0.9). The concentration of the small molecule was increased, and the inhibitory binding constant was determined (Ki_{app}). This is shown for two compounds (RU1 and RU46). In this competitive binding setup, the fluorescent polarization signal will drop as the fluorescently labeled peptide is competed off from the MAPK docking groove.

Figure 2A-B show the structure-activity relationship (SAR) analysis of cyclohexenone containing compounds binding to ERK2. Figure 2A shows the summary of competitive fluorescence polarization-based protein-peptide assays. The numbering of the carbon atoms of the cyclohexenone ring (1-6), the R'2 or R'4 substituent positions are indicated on the RU43 panel in the middle. Figure 2B shows that mutating Cys161 to alanine greatly decreases the binding affinity of RU64. Panels on the left show the direct binding titration with the proteins (wt or C161A) and the reporter D-peptide (fluorescently labeled), panels on the right show the results of the binding competition experiments.

Figure 3A-C show the reversibility of ERK2-cyclohexenone adduct formation. Figure 3A shows the intact mass before (left) and after dialysis (right) for ERK2-RU68. 5 µM ERK2 was mixed with 50 µM RU68 and analyzed by LC-MS, then the sample was dialyzed in buffer (20 mM Tris pH=8, 200 mM NaCl, 2 mM TCEP) overnight and analyzed by LC-MS again. Notice that the ratio of the ERK2 intact mass after the dialysis increases, and the ratio of the adduct decreases in the deconvoluted mass spectrum, indicating that RU68 adduct formation is reversible. Figure 3B shows real-time monitoring of ERK2-RU68 binding. ERK2 was covalently linked to the SPR chip surface (CM-5) by amine-coupling. RU68 was injected over this chip surface in 50 µM concentration for 10 minutes, then dissociation was followed for 20 minutes. Under these conditions the expected RUmax is ~45. Notice the complete dissociation of the small molecule by the end of the experiment, which is consistent with reversible binding. The panel shows the double-referenced binding curve using buffer injection correction against the experimental surface as well as sample injection correction against a control surface. Figure 3C shows the intact mass before (left) and after dialysis (right) for ERK2-RU66, -RU81, and RU77. 5 µM ERK2 was mixed with 500 µM compounds and analyzed by LC-MS, then the sample was dialyzed in buffer (20 mM Tris pH=8, 200 mM NaCl, 2 mM TCEP) overnight and analyzed by LC-MS again. Notice that the peak corresponding to the covalent adduct disappears in the deconvoluted mass spectrum, indicating that RU66, RU81 or RU77 adduct formation is also reversible.

Figure 4 shows the structural analysis of the ERK2-RU67 complex by X-ray crystallography. The lower panel on the left displays the Fo-Fc omit map shown at 2σ for the cysteine-RU67 covalent adduct. (ERK2 is shown in cartoon; the Cys161-RU167 adduct and the main chain of 159-163 are shown in sticks.) Panels on the right show the ERK2-RU67 crystallographic model. The docking groove is shown in surface representation and the Cys161-small molecule adduct with sticks. The lower panel also shows a D-peptide (pepMNK1, in gray) from the ERK2-pepMNK1 (PDB ID: 2Y9Q) overlayed, highlighting that the small molecule's cyclohexenone ring and the *tert*-butyl group bind to small hydrophobic pockets (φ_A and φ_B, respectively), while the bulky benzyl group faces towards φ_B. These small pockets are also used by proline, leucine, or methionine sidechains from the natural docking peptide (Garai et al, 2012).

Figure 5A-B shows the comparison of different MAPK D-groove (ERK2 Cys161) and JNK1 (Cys116) targeting composite inhibitors. Figure 5A shows the location of the two different cysteines: the D-groove cysteine found in all three MAPKs (ERK, p38, and JNK) and the unique cysteine (Cys116) in JNK1 located at its ATP-pocket/substrate binding pocket area. The surface presentation of the respective region around the cysteines (in darker gray) are shown in the lower panels from crystal structures (PDB ID: 2ERK, 5UOJ, 3V6S, and 2XRW). These show that Cys116 in JNK1 is more "open" and accessible at the wide substrate binding pocket compared to D-groove cysteines located in a "saddle" position. Figure 5B shows the measured apparent binding inhibitory values (Ki_{app}) of composite inhibitors comprised of two different directing groups (IN-8 scaffold for JNK1 Cys116 or *tert*-butyl group for ERK2 Cys161) and the same cyclohexenone warhead design connected via an amide or an ester. The upper panels for JNK1 show the

results of the NanoBRET target engagement assay (Reaction Biology Corp, USA). Duplicate measurements, error bars show SD. Lower panels for ERK2 show the results of in vitro fluorescence polarization (FP) based binding assays. Triplicate measurements, error bars show SD.

Figure 6A-D show the extension of the cyclohexenone scaffold towards the CD-groove by click chemistry. Figure 6A shows the crystal structure of the ERK2-pepMNK1 crystallographic complex (PDB ID: 2Y9Q; top) and the model of the in silico docked (hypothetical) RU83-methyl-triazole molecule (bottom). On the upper panel the arrow shows the position of proline at φ_L, whole the arrow on the lower panel shows the adjacent CD-groove. Figure 6B shows how the CD-groove binding C-terminal half of the MNK1 peptide with N-terminal azide (N_3-pepMNK1_C) was connected to RU83 containing an alkyne group at R'4 by copper-catalyzed azide/alkyne cycloaddition (^{t}Bu: *tert*-butyl group). Figure 6C shows the summary of competition binding experiments: MAPK binding affinity (K_i) of the RU83-click-pepMNK1_C chimera compared to RU83, N_3-pepMNK1_C, and pepMNK1 was measured using a fluorescence polarization-based assay, shown for two examples, with p38α and RU83 or RU83-click-pepMNK1_C (highlighted in bold font in the table) on the panels to the right; FB: fraction bound; *: competition is incomplete, Ki_{app} is only estimated. Figure 6D shows the results of SPR experiments with a p38α sensor chip. Sensorgrams show the binding of pepMNK1 or the RU83-peptide chimera injected under saturating conditions, in 20 μM or 30 nM concentration, respectively. Arrows indicate the start of the association or the dissociation phase. (RU: response units)

Figure 7A-C show the extension of the cyclohexenone warhead scaffold towards the ATP binding pocket. Figure 7A shows the structural model of JNK1 with a RU compound (RU60) bound in the D-groove and IN-8 in the ATP binding pocket. Figure 7B shows the results of equilibrium binding SPR experiments in the presence of 1 mM GSH. Figure 7C shows the kinetics of binding for IN-8 and RU128 injected over the JNK1 surface in a concentration corresponding to their equilibrium binding affinity (K_D). (Injection starts at time 0 and lasts for 5 minutes.)

Figure 8A-B show the cyclohexenone scaffold as a D-groove cysteine anchor to obtain MAPK-specific inhibitors in the presence of 10 mM GSH in vitro. Figure 8A shows how the different stereoisomers of a RU compound at C4 (e.g., RU83 or RU188) could be used to direct additional moieties towards the CD-groove or to the ATP binding pocket via click chemistry. The structure shows a model of the ERK2-RU60-ATP complex with pepMNK1_C. Figure 8B shows the summary of MAPK IC_{50} data determined by the PhALC assay (D-SENSOR, MEF2A) in the presence of 10 mM GSH. The panels below show two examples of the PhALC measurements with JNK1 and RU188 or RU128, highlighted in bold font in the table. (Error bars show the SD of three independent experiments.)

Figure 9A-C show the characterization of different compounds in cell-based tests. Figure 9A shows the characterization of RU83 and RU188 extended with a *N,N*-dimethylaniline moiety (RU103 and RU189) and tested in HEK293T using the NanoBiT protein-protein interaction (PPI) assay. ERK2 binding partners (MKK1, RSK1, and MKP3) interact via the MAPK D-groove ("-" indicates no inhibitor was added). The NanoBiT PPI assay detects higher level of binary protein binding interference in the presence of RU103 compared to its enantiomer (+RU103 vs +RU189). The NanoBiT luciferase fragment-complementation assay was done in live cells using 10 μM inhibitor concentration and 1.5-hour compound pre-treatment before the measurements. The panel on the left shows the results with an MKK1_D0 construct that did not contain the D-groove binding docking motif and was used as a control to demonstrate that the effect of RU103 on MKK1-ERK2 binding is D-groove dependent. (***: $p < 0.001$, NS: nonsignificant; two-sided, unpaired t-test; N=4). Figure 9B shows the effects of RU103 and RU189 on EGF-stimulated HeLa cells. Cells were nontreated (contr) or pretreated with 10 μM RU83 or RU189 (+RU83 or + RU189) for 1.5 hour and stimulated with 100 ng/mL EGF. The impact of the inhibitors on ERK or RSK phosphorylation was monitored by quantitative western blots using phosphospecific antibodies (ppERK or pRSK), and the signal divided by the WB signal of ERK or Tubulin (TUB) antibodies, respectively. These were then further normalized to the maximum signal (at 7 minutes for ppERK/ERK and 10 minutes for pRSK/TUB). Error bars indicate standard deviations (N=2). Figure 9C shows the results of an AP-1 promoter mediated transcription assay in HEK293 cells. The cell line contains an AP-1 promoter+luciferase transgene cassette stably integrated into the genome. Cells were unstimulated (-) or stimulated by Phorbol 12-myristate 13-acetate (+ PMA). Different inhibitors were added together with 10 nM PMA and the measurements were carried out after 6 hours. PMA-induced luciferase reporter gene transcription was monitored by luminescence measurements. In the first panel inhibitors (RU77, RU103 or RU189) were co-administered with PMA and were used in 1, 3, or 10 μM concentrations. (Error bars show standard deviation, N=3). Notice that RU77 shows significant inhibition at 3 μM concentration and above, while RU103 and RU189, which are enantiomeric pairs with extended moieties at C4, appear to be stronger inhibitors in this MAPK dependent cell-based test, however, RU103 performs significantly better at all three concentrations tested. The panel below shows the results of another experiment where the effects of RU215 and RU141, containing *N,N*-dimethylaniline or pyridine moiety, respectively (but C4 was extended by the same chemical solution, i.e. Sonogashira coupling). Note that RU103, RU141, and RU215 are all *S* stereoisomers at C4 but they contain different moieties (*N,N*-dimethylaniline or pyridine) or different chemical solutions for C4 extension (1,4-substituated 1,2,3-triazole: nonlinear or arylalkyne: linear). Error bars show SD (N=3). (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$; two-sided, unpaired t-test)

Figure 10A-B show the structural comparisons of the ERK2 covalent adducts with different cyclohexenone containing compounds. Figure 11A shows the crystallographic complexes ERK2-RU67 or -RU68, ERK2-RU75 or RU76, and ERK2-RU60 or RU187. ^{4}S or ^{4}R refer to the absolute configuration at C4; this is also shown with an arrowhead in the first panel, as well as the new asymmetric centers forming upon adduct formation at C2 and C3). RU67/RU68, RU75/RU76, and RU60/RU187 are enantiomeric pairs. The cysteine adduct in the ERK2-RU75 complex has two alternative C-S covalent bond conformations. The ERK2 D-groove is shown with surface representation from the same view for RU67/RU68, RU75/RU76, and RU60 (position of φ_B in the D-groove is shown to show the orientation and Cys161 is pointed at with an arrowhead). The crystal structure of the ERK2-RU187 complex is shown from a different angle. This complex has a cysteine as well as a histidine adduct (and the D-groove is shown in surface representation apart from the amino acid adducts, which are shown in sticks). Naturally, one molecule can form only one of the two adducts, and the final structural model fitted best with the crystallographic data if the two alternative adducts were equally present. (The conformation of the intact cysteine or the histidine sidechain is shown with thinner lines.) Figure 10B shows the Fo-Fc omit maps contoured at 2σ around Cys161 and His125 from the ERK2-RU60 and ERK2-RU187 crystal structures. The red sphere in the ERK2-RU187 complex shows a water molecule H-bonded with the His125-RU187 adduct and with the protein backbone (Ser122).

Figure 11A-B show the crystal structure of the ERK2-RU77 complex with the histidine adduct and the biochemical validation of histidine-adduct formation in the MAPK D-groove. Figure 11A shows the crystal structure of the ERK2-RU77 complex with the Fo-Fc omit map shown at 2σ for the histidine-small molecule covalent adduct. The panel on right shows the MAPK docking groove in surface representation and highlights the position of the different hydrophobic pockets (φ_A, φ_B, φ_L) and the polar CD groove. Figure 11B shows the results of the NanoBiT luciferase fragment-complementation assay. The assay was done in live HEK293T cells using 10 μM inhibitor (RU77) concentration and 1.5-hour compound pretreatment before the measurements. Panels show the NanoBiT luminescence signal for the MKK1-ERK2 interaction (left), where MKK1_D0 is a construct that lacks the first disordered 19 amino acids, containing the D-motif, of MKK1, and used to show that this interaction is D-motif dependent or for the MKK6-p38α interaction, also D-motif dependent (p values; two-sided, unpaired t-test; N=4).

Figure 12 shows the crystal structure of composite inhibitors in complex with JNK1. The BD837/BD838 warheads are enantiomers, and this structural comparison demonstrates that the C4 stereogenic center (pointed at with arrows for BD837/BD838 below) could be used to direct the carboxymethyl group towards different directions in the substrate binding pocket next to Cys116. Comparison of the JNK1–RU135-IN-8 and –BD837-IN-8 complexes shows that an additional methylene (highlighted with "*") between the ATP pocket binding moiety and the warhead necessitates a dramatically different conformational solution to form the cysteine covalent adduct. Lower panels display the Fo-Fc omit map for the cysteine-small molecule covalent adduct contoured at 1.5σ.

Figure 13A-D show the comparative binding energetics, and "residence time" enhancement (i.e. k_{off} decrease) due to reversible covalent bond formation for different Cys116 targeting JNK inhibitors. Figure 13A shows the scheme of the 2-step reversible kinetic binding model with the relevant kinetic binding constants for noncovalent binding via the IN-8 ATP-binding moiety (k1 and k2) as well as for warhead mediated covalent bond formation (k3 and k4) and the structure of the examined compounds. ($^{\#}$Note that RU213 and RU211 are enantiomers of RU212 and RU210, respectively). Figure 13B shows the SPR kinetic binding curves of two selected inhibitors (RU155-IN-8 and BD837-IN-8) as examples for the full analysis that was carried out for all the compounds shown on panel A (see Table 8). The experimental kinetic binding plots collected at three different analyte concentration, chosen according to the compounds's K_D , are shown in black and the calculated plots (shown in gray) are based on the 2-step reversible kinetic binding scheme with the final k1, k2, k3 and k4 values. (For some of the compounds with he fastest on-rates the kinetic binding curve corresponding to the highest concentration injection was not used in the the numerical fit, for example in the case of RU155-IN-8.) Figure 13C shows the kinetic binding plots for JNK-IN-8 on the JNK1(Cys116Ser) mutant surface injected at 100, 300, 1000 nM concentrations (K_D ~ 200-300 nM) as well as the response curves on the wild-type JNK1 surface (WT) at three different concentrations. The analysis on the JNK1(Cys116Ser) mutant surface gives the value of k1 and k2 based on a one-site noncovalent binding model; these values were independently determined for each inhibitor this way (see Table 8). Figure 13D shows binding energy contribution comparisons due to covalent bond formation through the respective warhead (ΔΔG) and the comparison of off-rates (k2: noncovalent in gray vs k4: covalent in black). Notice that JNK1-RU135-IN-8 covalent bond has ~100-fold increased off-rate compared to the more optimal conformation observed with the BD837 warhead. Moreover, a substantial gain in binding energetics as well as in on-site "residence" could be achieved by electronically tuning this suboptimal warhead by an ester instead of an amide at C2 (compare RU137-IN-8 with RU135-IN-8, respectively). Finally, residence of the inhibitor (~ pk4) is also affected by further sterical crowding at C4 (RU210 vs BD837) or by decreasing the flexibility of the cyclohexenone ring with the C4-C6 bridge (RU212 vs BD837). In the case of the irreversible JNK-IN8 inhibitor k4 = 0 (and thus only k3 was fit), while for the reversible inhibitors k3 and k4 were both numerically fit to the experimental kinetic curves obtained on the JNK1 WT surface.

Figure 14A-B show the results of a stability assay in a nucleophile rich environment, in the presence of 10 mM GSH. Figure 14A shows the inhibitory potential of JNK-IN-8 and BD837-IN-8 after pre-incubating the compounds for

different amounts of time in 10 mM GSH before adding them into the PhALC assay. Figure 14B shows the electrospray mass spectrometry characterization of JNK-IN-8 and BD837-IN-8 incubated in 10 mM GSH. The GSH-adduct formation is apparent in the different elution profile for JNK-IN-8 incubated with GSH for 18 hours vs no incubation (0 hr) as well as in the ion-chromatograms (RT: retention time; M^+, M^{2+}, M^{3+}indicate the mass of differently charged molecule ions). BD837-IN-8 stays intact and does not form an irreversible GSH adduct.

Figure 15A-E show the effects of JNK-IN-8 and BD837-IN-8 in cells on c-Jun phosphorylation. Figure 15A shows the impact of the inhibitors on sorbitol stimulated JNK and c-Jun phosphorylation in HEK293T cells. Cells were stimulated with sorbitol for 15 minutes which gives robust JNK activation. "-" indicates no treatment with inhibitors and "+DMSO" indicates treatment with DMSO used in the same amount (0.1 %) as an organic solvent for the inhibitors. Inhibitors were added 2 hours before stimulation. Note the changed electrophoretic mobility of JNK in the presence of JNK-IN-8, indicating irreversible JNK–JNK-IN-8 binding (compare lane 4-5 with the other lanes). (p-JNK: western blot, WB, signal with phosphoJNK antibody; p-c-Jun: WB with phospho-c-Jun (Ser73) antibody where MAPK mediated c-Jun phosphorylation results in differently phosphorylated phospho-c-Jun species with different electrophoretic mobility; the fastest migrating band is the least phosphorylated; Tubulin: WB with anti-tubulin antibody which serves as the load control; M: molecular marker). Figure 15B shows the effects of JNK-IN-8 and BD837-IN-8 on c-Jun phosphorylation in an engineered neuroblastoma cell line (SH-SY5Y MKK7 ACT). Phosphorylation of JNK (p-JNK) was stimulated by 2 or 6 hours of doxycycline (DOX) treatment. Figure 15C shows the EC_{50} of JNK-IN-8 and BD837-IN-8 on JNK mediated c-Jun phosphorylation in SH-SY5Y MKK7 ACT cells stimulated with doxycycline (DOX) for 6 hours (N=3; error bars show SD calculated based on three independent measurements). The phosphorylation of c-Jun was determined by using quantitative western blots (WB) using a phospo-c-Jun specific antibody (p-c-Jun). Tubulin antibody was used as the load control and the p-c-Jun signal was divided by the tubulin signal, and the latter was normalized to p-c-Jun/tubulin measured in cells that were not treated by any inhibitor. The lower panel shows a more detailed example of this type of analysis with BD838-IN-8. Control: cells were not treated with doxocycline (DOX) or inhibitor but only with 1% DMSO. The concentration of the used inhibitor is shown above the western blot image (from 30 μM to 10 nM) and the panel below shows the fit to the dose-response equation used to obtain the EC_{50} value of inhibitors (in this example based on one experiment, N=1). Figure 15D shows the same analysis but for RU155-IN-8 based on three independent experiments (N=3; error bars show SD.) Figure 15E show the results of short-term effects of JNK inhibitors in SH-SY5Y MKK7 ACT cells on c-Jun phosphorylation upon doxycycline (DOX) treatment for 2 or 4 hours in the absence (D2hr or D4hr, respectively) or in the presence of 0.7 μM DB837-IN-8 or JNK-IN-8. (Error bars indicate the SD calculated based on three independent experiments; p-values were calculated with a two-sided, paired t-test; "M" indicates the lanes with molecular weight markers.)

Figure 16A-B show the effects of JNK inhibitors on cell viability and AP-1 promoter driven transcription. Figure 16A shows the effect of inhibitors on JNK mediated cell death in SH-SY5Y MKK7 ACT cells. Endogenous JNK activation was initiated artificially by the addition of 2 μg/mL doxycycline (DOX) to engineered SH-SY5Y neuroblastoma cell line (in which the expression of an active MLK3-MKK7 chimera is controlled via the DOX dependent Tet-ON system). Cell viability was measured by monitoring the reducing power of living cells. The latter is a cell health indicator and can be monitored by fluorescence intensity measurements after addition of a resazurin-based solution (PrestoBlue). The first panel shows the linearity of the fluorescence signal with the cell number of untreated SH-SY5Y MKK7 ACT cells (Error bars show SD, N=2). The panel below shows the results of an experiment where cells were uninduced (Control) or induced doxycycline (+DOX) and treated with no inhibitor (DMSO) or with different inhibitors in 1 μM concentration. Cell viability was measured 72 hours after doxycycline and inhibitor co-administration. Notice that doxycycline treatment reduces cell viability but cells are well-protected by composite inhibitors containing covalent warheads (JNK-IN-8, BD837-IN-8, or BD838-IN-8). The panel to the right shows the results of another experiment focusing on RU159_isoPHEN and the comparison of its effect to that of IN-8 and JNK-IN-8 (1 μM inhibitor concentration as in the other experiment but more cell death was initiated with doxycycline). The panel above shows the corresponding phospho-JNK western blot confirming JNK activation upon doxycycline treatment (+DOX; the two different bands on the phospho-JNK western blot correspond to different JNK isoforms). (Error bars show SD, N=3; p-values were calculated based on two-sided, unpaired t-test; NS: not significant, $p > 0.05$.) Figure 16B shows the effect of inhibitors on JNK mediated AP-1 transcription factor promoter activity. Reporter AP-1–HEK293 Recombinant Cell Line was unstimulated (-) or stimulated with phorbol 12-myristate 13-acetate (+PMA) and AP-1 promoter driven transcription of the luciferase reporter gene was monitored by measuring luminescence after 6 hours. Inhibitors were co-administered with PMA and were used in 1, 3, or 10 μM concentrations. (Error bars show standard deviation, N=3). ("RU159_isoPHEN" denotes RU159-IN-7_isoPHEN.)

Figure 17A-C show the specificity of BD837-IN-8. Figure 17A shows the specificity of BD837-IN-8 on JNK1 in comparison to ERK2 and p38α in the PhALC assay. (Error bars show SD based on three experiments). Figure 17B shows that JNK inhibitors do not affect the p38 MAPK pathway. HEK293T MKK6EE cells (allowing p38-specific activation by doxycycline inducible expression of the constitutively active upstream activator kinase, MKK6EE, with a FLAG-tag) were stimulated for the indicated time by doxycycline (DOX) and cells were simultaneously treated with SB202190 (1 μM), a known p38-specific ATP-competitive inhibitor, or with different JNK inhibitors (3 μM). The upper panel shows the western blot results with a phospho-MK2 specific antibody, while the lower panel shows the

western blot results using three antibodies (anti-tubulin – as the load control, anti-pp-p38 – recognizing the activated form of p38 kinases, and anti-FLAG – monitoring the expression level of MKK6EE-FLAG). MK2, also known as MAPKAPK2, is a specific substrate of p38. Figure 17C shows that BD837-IN-8 does not interfere with docking peptide (EvJIP1) binding. A fluorescence polarization-based protein-peptide binding assay was used to detect the binding of the fluorescently labeled EvJIP1 peptide in the JNK docking groove harboring Cys163 (FB: fraction bound). The panel on the left shows how the unlabeled EvJIP1 peptide competes the labeled peptide off, and the panel on the right shows that the inhibitor does not interfere with docking motif binding, and thus demonstrating that this inhibitor does not target Cys163 located in the JNK docking groove.

Figure 18 shows the Specificity of BD837-IN-8 in the human kinome panel. Results of the Wild Type Kinase Panel (Reaction Biology Corp, USA; 340 human kinases) with BD837-IN-8 used at 1 μM concentration. Inhibition of a specific kinase is depicted with a circle on the human kinome tree, where circle size correlates with the amount of inhibition (and kinases are grouped based on sequence similarity into 7 kinase groups). The panels below show the structural models of LIMK1 (PDB ID: 3S95) and TNK1 (homology model created by AlphaFold 2) superimposed with the JNK1–BD837-IN-8 crystallographic model. LIMK1 and TNK1 are the only two human kinases whose activity were inhibited more than 50% (see Table 10).

Figure 19A-B show JNK isoform specificity of cyclohexenone warhead containing inhibitors. Figure 19A shows the crystal structure of the JNK1–BD837-IN-8 complex highlighting residues corresponding to exon 6 (shown with stick representation). This short region varies among JNK isoforms, and it forms the base of the substrate binding cleft next to the active site (D151). The three residues (GGV) displaying the greatest variation among the examined JNK isoforms are shown with spheres on the structural panel. Note that the substrate binding cleft is remodeled upon JNK activation loop (AL) phosphorylation, but the region corresponding to exon 6, especially the residues shown with spheres, will stay in the proximity of the cyclohexenone warhead. Inhibitor contacts will likely be affected by the stereochemistry of the warhead and/or the length of the substituent groups at C4. Figure 19B shows examples of PhALC assay measurements with RU144-IN-8 (see Table 11). Error bars show SD based on three independent experiments.

Figure 20A-C shows the effects of JNK PROTACs (RU219, RU231, RU232) on the level of JNK1 in HeLa cells. Figure 20A shows the structure of three PROTACs with the same BD837-IN-8 based JNK specificity element linked to two different ubiquitin ligase binding (VHL or CRBN) moieities using two different linkers. Figure 20B shows western blots results of three parallel experiments (1,2,3). The levels of JNK1 and p38α were monitored using JNK1- or p38α-specific antibodies and a tubulin-specific antibody was used as the load control (TUB). Cells were untreated ("Contr".), treated with one of the PROTACs, with a proteosome inhibitor (MG132), or with both (PR+MG132). JNK-specific PROTACS and MG132 were used in 10 μM concentration and treatment was for 24 hours. Figure 20C shows the quantitative summary of normalized JNK1 level changes or the p38α/TUB WB signal ratio. The JNK1 levels were normalized to the JNK1/TUB WB signal ratio of the "Contr". Error bars show SD based on three independent experiments (*: $p < 0.05$; two-sided, unpaired t-test).

Figure 21 shows the results of cell proliferation experiments with different human cell lines with different Ras GTPases (A549 with RaS_G12S mutant, H1792 with Ras_G12C mutant, or LCL-103H wild-type with no mutation at position 12). Cells were incubated with sc_AMG-510 or with RU230 (where the cyclohexenone warhead is indicated with a gray box) in 200 μM concentration for 72 hours. Cell viability was normalized to that of control cells that were not treated with any of the compounds. Error bars show SD based on three independent experiments (N=3). (NS: not significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$; two-sided, unpaired t-test). Cell viability measurments were taken at cell numbers which fall within the linear range of detection for all cell lines. This is shown for experiments with the H1792 cell line at the bottom panel. (Error bars show SD based on three indpenedent experiments; the dashed line shows linear regression.)

Definitions

Besides the compounds of Formula (I), pharmaceutically acceptable salts, solvates, hydrates, polymorphs, co-crystals, tautomers, stereoisomers, mixture of stereoisomers, e.g. racemates, diastereomers, enantiomers, mixtures of enantiomers, isotopically labeled derivatives, and prodrugs thereof belong to the scope of the present invention.

As used herein, the term "halo" or "halogen" means fluorine, chlorine, bromine or iodine, preferably fluorine, chlorine or bromine, even more preferably fluorine or chlorine.

As used herein, the term "alkyl" alone or in combinations means a straight or branched-chain saturated hydrocarbon group containing from 1 to 6, preferably 1 to 5 carbon atom(s) (i.e. "C_{1-6}" or "C_{1-5}" alkyl groups), such as methyl, ethyl, propyl, isopropyl, butyl, sec-butyl, tert-butyl and pentyl. In an embodiment, this phrase can relate to alkyl groups containing from 1 to 4, or 1 to 3, or 1 to 2 carbon atom(s) (i.e. "C_{1-4}" or "C_{1-3}" or "C_{1-2}" alkyl groups), where the methyl or ethyl is a preferred embodiment.

As used herein, the term “alkenyl” alone or in combinations means a straight or branched-chain hydrocarbon group containing from 2 to 6 carbon atoms or 2 to 5 carbon atoms, one or more carbon-carbon double bonds, and no triple bonds (i.e. “C_{2-6}” or “C_{2-5}” alkenyl groups). In an embodiment, this phrase can relate to alkenyl groups containing from 2 to 4, or 2 to 3 (i.e. “C_{2-4}” or “C_{2-3}” alkenyl groups). Examples are ethenyl, 1-propenyl, 2-propenyl, 1-butenyl, 2-butenyl, butadienyl, pentenyl, pentadienyl, hexenyl, and the like.

As used herein, the term “alkynyl” alone or in combinations means a straight or branched-chain hydrocarbon group containing from 2 to 6 carbon atoms or 2 to 5 carbon atoms, one or more carbon-carbon triple bonds, and optionally one or more double bonds (i.e. “C_{2-6}” or “C_{2-5}” alkynyl groups). In an embodiment, this phrase can relate to alkynyl groups containing from 2 to 4, or 2 to 3 (i.e. “C_{2-4}” or “C_{2-3}” alkynyl groups). Examples are ethynyl, 1-propynyl, 2-propynyl, 1-butynyl, 2-butynyl, pentynyl, hexynyl, and the like.

As used herein, the term “haloalkyl” means an alkyl group as defined above where one or more of the hydrogen atoms are substituted with halogen atoms.

In an embodiment alkyl, alkenyl and alkynyl are unsubstituted. In a further embodiment, alkyl, alkenyl, and alkynyl are independently substituted with one or more substituents selected from the group consisting of -CN, -NO_2, -NH_2, -OH, -SH, -CO_2H, -CHO.

The term “aralkyl” refers to an alkyl group substituted with an aryl group.

The term "alkoxy" refers to an alkyl group, as defined above, attached to the rest of the molecule through an oxygen atom.

The term “amino” means an optionally substituted -NH_2 group. In an embodiment, amino is substituted with one or two alkyl, preferably methyl or ethyl.

The term “hydroxyl” means an -OH group.

As used herein the term “carbocyclyl”, alone or in combinations means a saturated or partially unsaturated cyclic hydrocarbon group having from 3 to 10 ring carbon atoms ("C_{3-10} cycloalkyl") and zero heteroatoms in the non-aromatic ring system. In special cases, this phrase can relate to carbocyclyl groups containing from 3 to 8, or 3 to 6 (i.e. “C_{3-8}” or “C_{3-6}” cycloalkyl groups). Non-limiting examples are cyclopropyl, cyclopropenyl, cyclobutyl, cyclobutenyl, cyclopentyl, cyclopentenyl, cyclohexyl, cyclohexenyl, cyclohexadienyl, cycloheptyl, cycloheptenyl, cycloheptadienyl, cycloheptatrienyl, cyclooctyl, cyclooctenyl, bicyclo[2.2.1]heptanyl, bicyclo[2.2.2]octanyl, cyclononyl, cyclononenyl, cyclodecyl, cyclodecenyl, octahydro-1*H*-indenyl, decahydronaphthalenyl, and spiro[4.5]decanyl. The carbocyclyl group is either monocyclic or contains a fused, bridged, or spiro ring system such as a bicyclic system. "Carbocyclyl" also includes ring systems wherein the carbocyclic ring, as defined above, is fused with one or more aryl or heteroaryl groups wherein the point of attachment is on the carbocyclic ring, and in such instances, the number of carbons continue to designate the number of carbons in the carbocyclic ring system. Preferred carbocyclic groups are cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl.

As used herein the term "heterocyclyl" alone or in combinations means a saturated or partially unsaturated non-aromatic ring system having 4 to 9 ring carbon atoms and 1 to 4 ring heteroatoms, wherein each heteroatom is independently selected from nitrogen, oxygen, sulfur, boron, phosphorus, and silicon ("3-10 membered heterocyclyl"). In special cases, this phrase can relate to heterocyclyl groups having 3-8 ring forming atoms ("3-8 membered heterocyclyl") or 4-6 ring forming atoms ("4-6 membered heterocyclyl"). In heterocyclyl groups that contain one or more nitrogen atoms, the point of attachment can be a carbon or nitrogen atom, as valency permits. A heterocyclyl group can either be monocyclic ("monocyclic heterocyclyl") or a fused, bridged or spiro ring system such as a bicyclic system ("bicyclic heterocyclyl”). Non-limiting examples are azirdinyl, oxiranyl, thiorenyl, azetidinyl, oxetanyl, thietanyl, tetrahydrofuranyl, dihydrofuranyl, tetrahydrothiophenyl, dihydrothiophenyl, pyrrolidinyl, dihydropyrrolyl, pyrrolyl-2,5-dione, dioxolanyl, oxasulfuranyl, disulfuranyl, oxazolidin-2-one, triazolinyl, oxadiazolinyl, thiadiazolinyl, piperidinyl, tetrahydropyranyl, dihydropyridinyl, thianyl, piperazinyl, morpholinyl, dithianyl, dioxanyl, triazinanyl, azepanyl, oxepanyl, thiepanyl, azocanyl, oxecanyl, thiocanyl, indolinyl, isoindolinyl, dihydrobenzofuranyl, dihydrobenzothienyl, benzoxazolinonyl, tetrahydroquinolinyl, tetrahydroisoquinolinyl, and the like. Heterocyclyl bicyclic ring systems can include one or more heteroatoms in one or both rings. "Heterocyclyl" also includes ring systems wherein the heterocyclic ring, as defined above, is fused with one or more carbocyclyl groups wherein the point of attachment is either on the carbocyclic or heterocyclic ring, or ring systems wherein the heterocyclic ring, as defined above, is fused with one or more aryl or heteroaryl groups, wherein the point of attachment is on the heterocyclic ring, and in such instances, the number of ring members continue to designate the number of ring members in the heterocyclic ring system. Preferred heterocyclyl groups are azetidine, oxetane, pyrrolidine, tetrahydrofuran, piperidine, pyran, morpholine, oxazine, dioxane.

In an embodiment carbocyclyl and heterocyclyl are unsubstituted. In a further embodiment, carbocyclyl and heterocyclyl are independently substituted with one or more substituents selected from the group consisting of halogen, hydroxy, alkoxy, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted carbocyclyl, optionally substituted heterocyclyl, optionally substituted aryl, optionally substituted heteroaryl, optionally substituted amino.

As used herein the term “aryl”, alone or in combinations means an aromatic monocyclic or condensed double ring system comprising 6 to 10 carbon atoms. Nonlimiting examples of suitable aryl groups include phenyl, and naphthyl, where phenyl is a preferred embodiment.

As used herein, the term “isosteres” means groups with similar physical or chemical properties which produce broadly similar biological properties in the same chemical compound. Phenyl isosteres are for example pyridinyl, thiophenyl, cubane-1,4-diyl, bicyclo[2.2.2]-octane-1,4-diyl and bicyclo[1.1.1]-pentane-1,4-diyl, preferably bicyclo[1.1.1]-pentane-1,4-diyl.

The term “heteroaryl” means a group derived from a monocyclic or condensed double ring system with 1 to 3 ring forming heteroatom(s) selected from the group of N, O and S and 3 to 9 ring forming carbon atoms ("5-10 membered heteroaryl"). In special cases, this phrase can relate to heteroaryl groups having 5-8 ring forming atoms ("5-8 membered heteroaryl") or 5-6 ring forming atoms ("5-6 membered heteroaryl"). "Heteroaryl" includes ring systems wherein the heteroaryl ring, as defined above, is fused with one or more carbocyclic or heterocyclic groups wherein the point of attachment is on the heteroaryl ring, and in such instances, the number of ring members continue to designate the number of ring members in the heteroaryl ring system. "Heteroaryl" also includes ring systems wherein the heteroaryl ring, as defined above, is fused with one or more aryl groups wherein the point of attachment is either on the aryl or heteroaryl ring, and in such instances, the number of ring members designates the number of ring members in the fused (aryl/heteroaryl) ring system. Non-limiting examples are pyrrolyl, furanyl, thiophenyl, imidazolyl, pyrazolyl, oxazolyl, isoxazolyl, thiazolyl, isothiazolyl, triazolyl, oxadiazolyl, thiadiazolyl, tetrazolyl, pyridinyl, pyridazinyl, pyrimidinyl, pyrazinyl, triazinyl tetrazinyl, azepinyl, oxepinyl, thiepinyl, indolyl, isoindolyl, indazolyl, benzotriazolyl, benzothiophenyl, isobenzothiophenyl, benzofuranyl, benzoisofuranyl, benzimidazolyl, benzoxazolyl, benzisoxazolyl, benzoxadiazolyl, benzthiazolyl, benzisothiazolyl, benzthiadiazolyl, indolizinyl, purinyl, naphthyridinyl, pteridinyl, quinolinyl, isoquinolinyl, cinnolinyl, quinoxalinyl, phthalazinyl, and quinazolinyl. In heteroaryl groups that contain one or more heteroatoms, the point of attachment can be a carbon or heteroatom, as valency permits. Preferred heteroaryl groups are imidazole, pyrrole, pyrazole, oxazole, isoxazole, thiazole, isothiazole, furan, thiophene, pyridine.

In an embodiment aryl, phenyl isosteres and heteroaryl are unsubstituted. In a further embodiment, aryl, phenyl isosteres and heteroaryl are independently substituted with one or more substituents selected from the group consisting of halogen, amino, alkoxy, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted carbocyclyl, optionally substituted heterocyclyl, optionally substituted aryl, optionally substituted heteroaryl, optionally substituted amino. Preferred substituents are halogen, C_{1-4} or C_{1-3} alkyl, C_{1-4} or C_{1-3} haloalkyl, C_{2-4} or C_{2-3} alkenyl, C_{2-4} or C_{2-3} alkynyl, amino, (C_{1-4} or C_{1-3})alkylamino, di(C_{1-4} or C_{1-3})alkylamino, (C_{1-4} or C_{1-3}) alkoxy.

N-protecting groups are well known in the art and include those described in detail in Protecting Groups in Organic Synthesis, T. W. Greene and P. G. M. Wuts, 3rd edition, John Wiley & Sons, 1999. Preferred examples are acetyl, benzoyl, benzyl, BOC, FMOC, carbobenzyloxy, carbamate and tosyl group.

A "chemically reasonable radical" means in the context of the present invention a molecular fragment which is stable at room temperature and atmospheric pressure. In the context of the present invention, a "chemically reasonable radical" in compounds of the formula I preferably means that bonding individual members of the list does not result in a chain moiety containing more than 2 heteroatoms (i.e. moieties such as -O-O-O- are excluded).

“Frustration” refers to sterical crowding effects by substituents on a moiety around the reactive center, specifically on a cycloalkenone moiety as used herein, which can block the formation of adducts with hindered amino acid residues endowing higher site selectivity for the ligands and mitigating “off-target” binding. Additionally, the same steric effect can result in the formation of a non-stable Michael adduct and facilitates the elimination of Michael-donor amino acid residues.

A “subject” as used herein is an individual of an animal species, preferably a vertebrate, more preferably a mammalian or avian species, in particular a mammalian species, highly preferably the individual is a primate, a hominid or a human.

A “patient” is a subject who is or intended to be under medical or veterinarian observation, supervision, diagnosis or treatment.

A "treatment" refers to any process, action, application, therapy, or the like, wherein the subject or patient is under aid, in particular medical or veterinarian aid with the object of improving or maintaining the subjects's or patient's condition, either directly or indirectly. Improving the subjects's condition may include restoring or maintaining normal function of an organ or tissue, preferably at least partly restoring or maintaining health (medical or veterinarian treatment). Treatment typically refers to the administration of an effective amount of a compound or composition described herein. Treatment may relate to or include medical or veterinarian treatment and cosmetic treatment, in particular medical or veterinarian treatment.

"Preventing" or "prevention" of the development of a disease or condition refers to at least the reduction of likelihood of the risk of or susceptibility to acquiring a disease or disorder, or preferably causing at least one of the clinical symptoms of the disease or disorder not to develop in a patient that may be exposed to or predisposed to the disease but does not yet experience or display symptoms of the disease.

A "neoplasm" is a type of abnormal and excessive growth of tissue. (The process that occurs to form or produce a neoplasm is called "neoplasia".) The growth of a neoplasm is uncoordinated with that of the normal surrounding tissue, and persists in growing abnormally, even if the original trigger is removed.
A malignancy is a malignant neoplasm.

The term "tumor" is used to describe a neoplasm, which is an abnormally increased mass of tissue or population of cells.

"Cancer", as used herein is a malignant tumor, and relates to a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body.

"Conservative" amino acids (preferably a cysteine or histidine) refer to amino acids found in related proteins in related positions. They share common biochemical properties for selective covalent targeting by an electrophile.
For example a conservative amino acid (preferably cysteine or histidine) of a protein family is understood herein as an amino acid (a cysteine or histidine) present in a given related position in the majority or most of the members of the protein family or in a subgroup.
Examples for such protein families are the mitogen-activated protein kinases (MAP kinases or MAPK) or MAPK subfamilies like ERK1, ERK2, JNK, p38 kinases, ERK5 etc.
In particular embodiment the related position is a position which is important for or has a role in the biochemical properties of the related proteins, e.g. throughout a given protein family or variant group of said protein family.

MAP kinases or MAPK are mitogen-activated protein kinases and the term are used interchangeably.
The terms extracellular regulated kinase 1 (ERK1) and ERK1 kinase are used interchangeably.
The terms extracellular regulated kinase 2 (ERK2) and ERK2 kinase are used interchangeably.
The terms extracellular regulated kinase 5 (ERK5) and ERK5 kinase are used interchangeably.
The terms c-Jun N-terminal kinase 1 (JNK1) and JNK1 kinase are used interchangeably.
The terms c-Jun N-terminal kinase 2 (JNK2) and JNK2 kinase are used interchangeably.
The terms c-Jun N-terminal kinase 3 (JNK3) and JNK3 kinase are used interchangeably.

Pharmaceutical Compositions and Administration

The present invention provides pharmaceutical compositions comprising a compound of the present invention, e.g., a compound of Formula (I), and pharmaceutically acceptable salts thereof, as described herein, and optionally a pharmaceutically acceptable excipient. In certain embodiments, the compound of the present invention, or a pharmaceutically acceptable salt thereof, is provided in an effective amount in the pharmaceutical composition. In certain embodiments, the effective amount is a therapeutically effective amount. In certain embodiments, the effective amount is a prophylactically effective amount.

Pharmaceutical compositions described herein can be prepared by any method known in the art of pharmacology. In general, such preparatory methods include the steps of bringing the compound of the present invention (the "active ingredient") into association with a carrier and/or one or more other accessory ingredients, and then, if necessary and/or desirable, shaping and/or packaging the product into a desired single- or multi-dose unit.

Pharmaceutical compositions can be prepared, packaged, and/or sold in bulk, as a single unit dose, and/or as a plurality of single unit doses. As used herein, a "unit dose" is a discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient which would be administered to a subject and/or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

Relative amounts of the active ingredient, the pharmaceutically acceptable excipient, and/or any additional ingredients in a pharmaceutical composition of the invention will vary, depending upon the identity, size, and/or condition of the subject treated and further depending upon the route by which the composition is to be administered. By way of example, the composition may comprise between 0.1% and 100% (w/w) active ingredient.

Pharmaceutically acceptable excipients used in the manufacture of provided pharmaceutical compositions include inert diluents, dispersing and/or granulating agents, surface active agents and/or emulsifiers, disintegrating agents, binding agents, preservatives, buffering agents, lubricating agents, and/or oils. Excipients such as cocoa butter and suppository waxes, coloring agents, coating agents, sweetening, flavoring, and perfuming agents may also be present in the composition. These ingredients are well known in the state of the art.

Dosage forms

The compounds and compositions of the invention can be administered by any route, including enteral (*e.g.*, oral), parenteral, intravenous, intramuscular, intraarterial, intramedullary, intrathecal, subcutaneous, intraventricular, transdermal, intradermal, rectal, intravaginal, intraperitoneal, topical (as by powders, ointments, creams, and/or drops), mucosal, nasal, buccal, sublingual; by intratracheal instillation, bronchial instillation, and/or inhalation; and/or as an oral spray, nasal spray, and/or aerosol. Specifically contemplated routes are oral administration, intravenous administration (*e.g.*, systemic intravenous injection), regional administration via blood and/or lymph supply, and/or direct administration to an affected site. The formulations appropriate for said administration routes are well known in the state of the art.

Dosage forms for injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions can be formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation can be a sterile injectable solution, suspension or emulsion in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol.

Compositions for rectal or vaginal administration are typically suppositories which can be prepared by mixing the conjugates of this invention with suitable nonirritating excipients or carriers such as cocoa butter, polyethylene glycol, or a suppository wax which are solid at ambient temperature but liquid at body temperature and therefore melt in the rectum or vaginal cavity and release the active ingredient.

Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active ingredient is mixed with at least one inert, pharmaceutically acceptable excipient or carrier and/or (a) fillers or extenders (b) binders, (c) humectants, (d) disintegrating agents, (e) solution retarding agents, (f) absorption accelerators, (g) wetting agents, (h) absorbents, and (i) lubricants, and mixtures thereof. In the case of capsules, tablets, and pills, the dosage form may comprise buffering agents.

Dosage forms for topical and/or transdermal administration of a compound of this invention may include ointments, pastes, creams, lotions, gels, powders, solutions, sprays, inhalants and/or patches. Generally, the active ingredient is admixed under sterile conditions with a pharmaceutically acceptable carrier and/or any needed preservatives and/or buffers as can be required. Additionally, the present invention contemplates the use of transdermal patches, which often have the added advantage of providing controlled delivery of an active ingredient to the body. Such dosage forms can be prepared, for example, by dissolving and/or dispensing the active ingredient in the proper medium. Alternatively, or additionally, the rate can be controlled by either providing a rate controlling membrane and/or by dispersing the active ingredient in a polymer matrix and/or gel.

Administration

The exact amount of a compound required to achieve an effective amount will vary from subject to subject, depending, for example, on species, age, and general condition of a subject, severity of the side effects or disorder, identity of the particular compound(s), mode of administration, and the like. Determination of the amount to be administered is within the competence of the skilled practitioner and represents an aspect of the state of the art.

Compounds according to the present invention can be used alone or in combination with other agents, wherein at least one compound according to the present invention is administered simultaneously, consecutively or prior to the other treatment.

Use

The present invention provides compounds for use in the prevention and treatment of various diseases, such as neurodegenerative diseases, e.g. Parkinson's disease, Alzheimer's disease, regeneration/repair after an injury to the

central nervous system, e.g. axonal regeneration, metabolic disorders, e.g. diabetes, inflammatory diseases, cardiovascular diseases, e.g. stroke and hypertension, and proliferative diseases, *e.g.*, cancer and benign neoplasms.

In certain embodiments, the disease is a disease associated with JNK activity, *e.g.,* a disease associated with aberrant or unwanted JNK activity. For example, in certain embodiments, the disease results from increased JNK activity. In certain embodiments, the compounds of the present invention, or the pharmaceutical compositions thereof are useful in the treatment of JNK-associated disease. Inhibition of JNK1 is associated with the treatment of cancer, diabetes, and inflammatory diseases (*e.g.*, inflammation). Increased JNK1 activity is also associated with obesity, *i.e.*, inhibition of JNK1 or mouse knockout has been found to increase insulin sensitivity (Hirosumi et al., 2002). Inhibition of JNK3 is associated with the treatment of neurodegenerative diseases. See Liu et al, 1998; Kyriakis et al., 2001; Zhang et al., 2005; and Hunot et al., 2004 for discussions of the association of JNK with various neurodegenerative diseases, *e.g.*, Huntington's, Parkinson's and Alzheimer's diseases.

In certain embodiments, the disease is a disease associated with ERK activity, *e.g.,* a disease associated with aberrant or unwanted ERK activity. In certain embodiments, the compounds of the present invention, or the pharmaceutical compositions thereof are useful in the treatment of ERK-associated disease, particularly in cancer (Smalley et al, 2018).

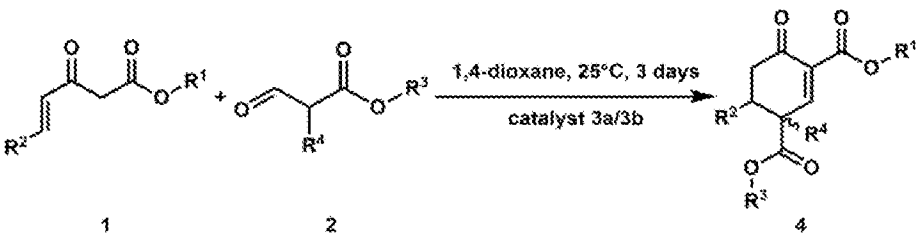
In certain embodiments, the disease is a disease associated with p38 activity, *e.g.,* a disease associated with aberrant or unwanted p38 activity. In certain embodiments, the compounds of the present invention, or the pharmaceutical compositions thereof are useful in the treatment of p38-associated disease, particularly in inflammation, immune disorders or cancer (Gupta et al., 2015) (Canovas et al, 2021).

Examples

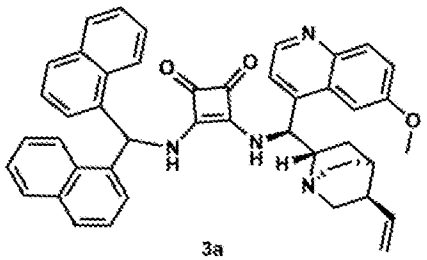
In order that the invention described herein may be more fully understood, the following examples are set forth. The synthetic and biological examples described in this application are offered to illustrate the compounds, pharmaceutical compositions, and methods provided herein and are not to be construed in any way as limiting their scope.

Example 1: Preparation of the Compounds

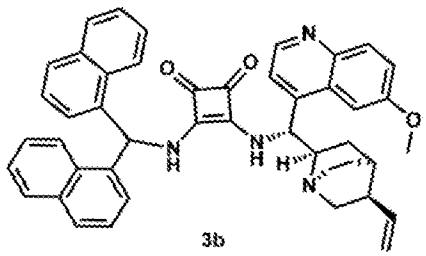
General procedure A for synthesizing cyclohexenone warhead compounds: (4)



1 2 4



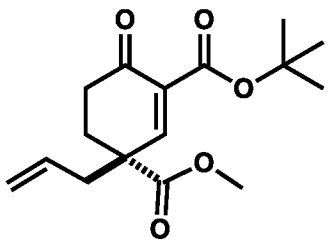
3a



3b

Compounds **1**, **2**, **3a**, **3b,** and **4** were synthesized according to literature procedures (Berkes et al., 2016) (Varga et al., 2020).

Preparation of 1-(*tert*-butyl) 3-methyl-3-allyl-6-oxocyclohex-1-ene-1,3-dicarboxylate: (RU41)

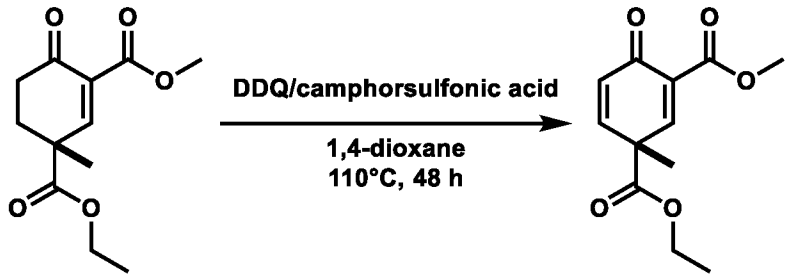


Following the **General Procedure A** using: *tert*-butyl 3-oxopent-4-enoate (14.3 g, 83.8 mmol), methyl 2-formylpent-4-enoate (13.1 g, 92.2 mmol) and triethylamine (2.3 mL, 16.8 mmol) in 1,4-dioxane (84 mL, 1.0 M) at room temperature for 2 days. After removal of the volatile compounds under reduced pressure, purification by flash chromatography on silica gel (hexanes 0-25% ethyl acetate) gave the product as a yellow oil (18.4 g, 75%).

LC-MS ESI $[M-(^tBu)+H^+]^+$= 239

^1H NMR: (500 MHz, $CDCl_3$) 7.35 (s, J = 4.7 Hz, 1H), 5.72 – 5.62 (m, 1H), 5.19 – 5.11 (m, 2H), 3.73 (s, 3H), 2.52 (ddt, J = 16.2, 11.3, 5.4 Hz, 4H), 2.47 – 2.34 (m, 1H), 2.03 – 1.95 (m, 1H), 1.51 – 1.49 (s, 9H).
^{13}C NMR: (125 MHz, $CDCl_3$) 193.7, 172.6, 163.7, 152.8, 133.9, 131.5, 120.2, 82.3, 52.7, 48.2, 42.8, 35.4, 29.8, 28.1;

3-ethyl 1-methyl 3-methyl-6-oxocyclohexa-1,4-diene-1,3-dicarboxylate: (RU44)

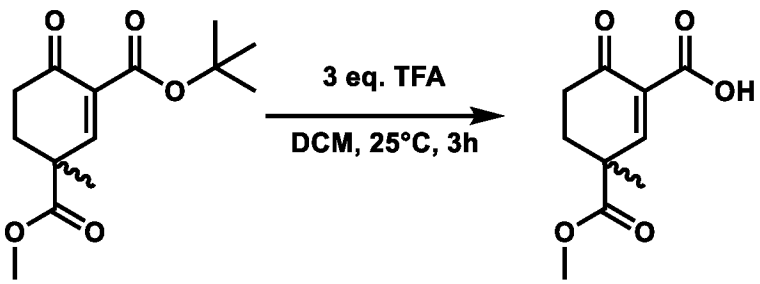


To a stirred solution of 3-ethyl 1-methyl 3-methyl-6-oxocyclohex-1-ene-1,3-dicarboxylate (1.01 g, 4.2 mmol) in anhydrous 1,4-dioxane (40 mL) were added 4Å molecular sieves and camphorsulfonic acid (0.54 g, 2.3 mmol). DDQ (1.16 g, 5.1 mmol) was added portionwise with vigorous stirring. The reaction mixture was heated to reflux and stirred for 48 hours. Upon completion, the mixture was cooled to 0°C, filtered through a pad of Celite, washed with 1,4-dioxane, and the solvent was evaporated under reduced pressure. The residue was dissolved in toluene, distilled water was added, then the pH was adjusted to 7-8 with a saturated $NaHCO_3$ solution. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, the solvent was removed under reduced pressure to give the product as a yellow oil (0.65 g, 65%).

LC-MS ESI $[M+H^+]^+$= 239

^1H NMR: (500 MHz, $CDCl_3$) 7.69 – 7.67 (m, 1H), 7.03 – 6.99 (m, 1H), 6.33 – 6.29 (m, 1H), 4.22 – 4.17 (m, 2H), 3.84 (s, 3H), 1.59 (s, 3H), 1.28 – 1.24 (m, 3H).
^{13}C NMR: (125 MHz, $CDCl_3$) 180.5, 169.7, 164.7, 153.6, 147.6, 131.7, 129.6, 62.7, 52.5, 48.4, 24.8, 14.0;

3-(methoxycarbonyl)-3-methyl-6-oxocyclohex-1-ene-1-carboxylic acid: (RU57)



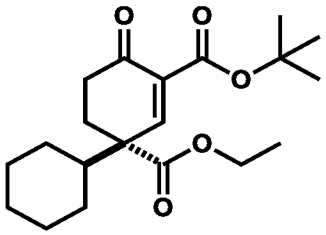
Trifluoroacetic acid (811 µL, 10.53 mmol, 3 eq.) was added to a solution of compound **4** (942 mg, 3.51 mmol, 1 eq.) in methylene chloride (20 mL) and then the mixture was stirred at 25°C. The reaction was monitored by TLC and when it

was completed (~3 hours) the reaction mixture was evaporated. The remaining trifluoroacetic acid was removed by redissolving the mixture in toluene and evaporating to dryness at 50°C three times. Compound **5** was obtained as a yellow oil in quantitative yield (745.0 mg) and was used in the next step without further purification.

LC-MS ESI $[M+H^+]^+$= 213;

^{1}H NMR (500 MHz, $CDCl_3$): 8.30 (s, 1H), 3.79 (s, 3H), 2.78–2.65 (m, 2H), 2.58–2.53 (m, 1H), 2.08–2.02 (m, 1H), 1.56 (s, 1H).
^{13}C NMR (125 MHz, $CDCl_3$): 200.1, 172.6, 165.2, 163.4, 126.0, 53.2, 45.4, 34.6, 31.8, 24.5;

3-(*tert*-butyl) 1-ethyl (S)-4-oxo-[1,1'-bi(cyclohexan)]-2-ene-1,3-dicarboxylate: (RU61)

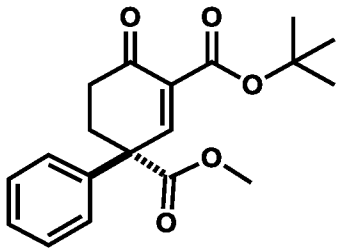


Following the **General Procedure A** using: *tert*-butyl 3-oxopent-4-enoate (85.1 mg, 0.50 mmol), ethyl 2-cyclohexyl-3-oxopropanoate (109 mg, 0.55 mmol), and DBU (15 µl, 0.100 mmol) in 1,4-dioxane (0.50 mL, 1.0 M) at room temperature for 2 days. After removal of the volatile compounds under reduced pressure, purification by flash chromatography on silica gel (hexanes 0-25% ethyl acetate) gave the product as a yellow oil (39 mg, 22%).

LC-MS ESI $[M-(^t Bu)+H^+]^+$= 295

^{1}H NMR: (500 MHz, $CDCl_3$): 7.49 (d, J = 1.7 Hz, 1 H), 4.28 (q, J = 7.1 Hz, 2 H) 4.26-4.13 (m, 2 H), 2.59- 2.48 (m, 2 H), 2.40-2.35 (m, 1 H), 2.04 (dt, J = 13.2, 5.3 Hz, 1 H), 1.90 (tt, J = 12.1, 2.9 Hz, 1 H), 1.83-1.77 (m, 2 H), 1.69 (d, J = 12.9 Hz, 1 H), 1.64-1.57 (m, 2 H), 1.33 (t, J = 7.1 Hz, 3 H), 1.28 (t, J = 7.1 Hz, 4 H), 1.24-1.01 (m, 4 H)
^{13}C NMR: (125 MHz, $CDCl_3$): 194.2, 172.5, 171.9, 163.8, 154.6, 133.8, 81.9, 61.4, 51.9, 45.9, 35.9, 28.0, 27.9, 27.7, 26.5, 26.0, 25.8, 14.1;

3-(*tert*-butyl) 1-methyl 4-oxo-5,6-dihydro-[1,1'-biphenyl]-1,3(4*H*)-dicarboxylate: (RU62)

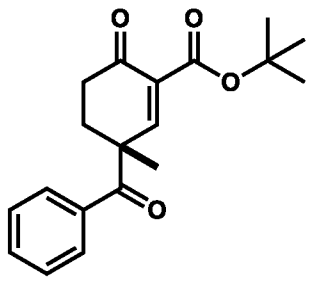


Following the **General Procedure A** using: *tert*-butyl 3-oxopent-4-enoate (85 mg, 0.5 mmol), methyl 3-oxo-2-phenylpropanoate (98 mg, 0.55 mmol), and triethylamine (14 µL, 0.1 mmol) in 1,4-dioxane (0.5 mL, 1.0 M) at room temperature for 2 days. After removal of the volatile compounds under reduced pressure, purification by flash chromatography on silica gel (hexanes 0-25% ethyl acetate) gave the product as a yellow oil (62 mg, 38%).

LC-MS ESI $[M-(^t Bu)+H^+]^+$= 275

^{1}H NMR: (500 MHz, $CDCl_3$) 7.79 (s, 1H), 7.40 – 7.35 (m, 2H), 7.34 – 7.29 (m,1H), 7.26 (s, 2H), 3.76 (s, 3H), 2.78 – 2.69 (m, 1H), 2.59 – 2.50 (m, 1H), 2.42 – 2.29 (m, 2H), 1.55 (s, 9H).
^{13}C NMR: (125 MHz, $CDCl_3$) 193.5, 172.0, 163.8, 152.0, 139.9, 135.2, 129.2, 128.1, 126.3, 82.5, 53.2, 52.7, 35.4, 33.4, 28.2;

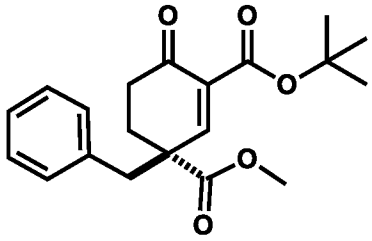
***Tert*-butyl (R)-3-benzoyl-3-methyl-6-oxocyclohex-1-ene-1-carboxylate: (RU63)**



Following the **General Procedure A** using: *tert*-butyl 3-oxopent-4-enoate (85 mg, 0.5 mmol), 2-methyl-3-oxo-3-phenylpropanal (89 mg, 0.55 mmol), and triethylamine (14 µl, 0.1 mmol) in 1,4-dioxane (0.5 mL, 1.0 M) at room temperature for 2 days. After removal of the volatile compounds under reduced pressure, purification by flash chromatography on silica gel (hexanes 0-25% ethyl acetate) gave the product as a yellow oil (72 mg, 46%).

^{1}H NMR: (500 MHz, $CDCl_3$) 7.77 – 7.72 (m, 2H), 7.56 (s, 1H), 7.54 – 7.49 (m, 1H), 7.47 – 7.39 (m, 2H), 2.70 – 2.62 (m, 1H), 2.56 (tdt, J = 17.1, 8.3, 4.3 Hz, 2H), 2.08 – 2.00 (m, 1H), 1.57 (s, 3H), 1.49 (s, 9H).
^{13}C NMR: (125 MHz, $CDCl_3$) 201.7, 193.6, 163.6, 155.5, 136.8, 132.5, 130.2, 128.6, 128.6, 82.3, 49.7, 35.3, 33.0, 28.1, 24.1;

1-(*tert*-butyl) 3-methyl-3-benzyl-6-oxocyclohex-1-ene-1,3-dicarboxylate: (RU67/RU68)

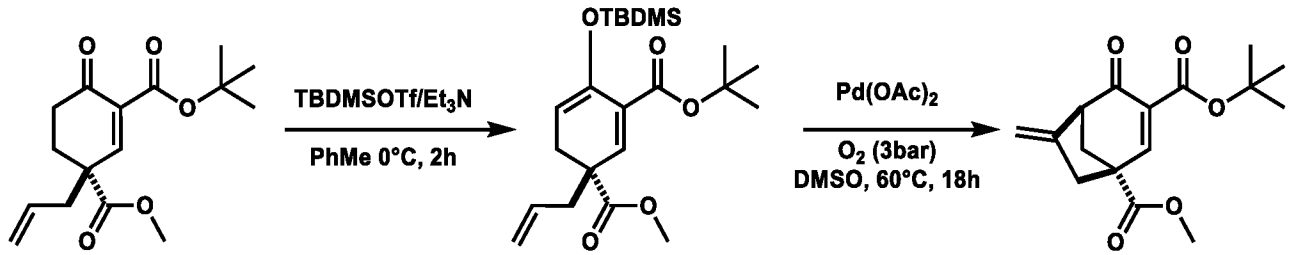


Following the **General Procedure A** using: *tert*-butyl 3-oxopent-4-enoate (85 mg, 0.5 mmol), methyl 2-benzyl-3-oxopropanoate (106 mg, 0.55 mmol), and triethylamine (14 µl, 0.1 mmol) in 1,4-dioxane (0.5 mL, 1.0 M) at room temperature for 2 days. After removal of the volatile compounds under reduced pressure, purification by flash chromatography on silica gel (hexanes 0-25% ethyl acetate) gave the product as a yellow oil (110 mg, 64%).

ee: 92% with catalyst **3a**
ee: -76% with catalyst **3b**
LC-MS ESI $[M-(^tBu)+H^+]^+$= 289

^{1}H NMR: (500 MHz, $CDCl_3$) 7.42 (s, 1H), 7.26 (m, 3H), 7.10 – 7.05 (m, 2H), 3.68 (s, J = 4.8 Hz, 3H), 3.10 (s, 2H), 2.51 – 2.47 (m, 2H), 2.42 – 2.33 (m, 1H), 2.08 – 2.00 (m, 1H), 1.51 (s, J = 4.6 Hz, 9H).
^{13}C NMR: (125 MHz, $CDCl_3$) 193.5, 172.5, 163.5, 153.0, 135.0, 133.6, 129.8, 128.5, 127.49, 82.1, 52,5, 49.4, 44.5, 35.3, 30.0, 28.1;

3-(*tert*-butyl) 1-methyl 6-methylene-4-oxobicyclo[3.2.1]oct-2-ene-1,3-dicarboxylate: (RU73/RU77)



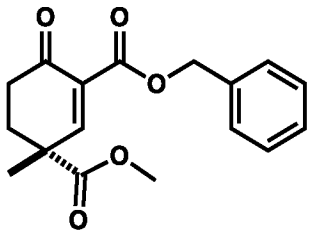
A flame-dried vial was charged with an anhydrous toluene solution (3.5 mL) of **RU41** (1.00 g, 3.40 mmol, 1 eq.) and triethylamine (940 µL, 6.79 mmol, 2 eq.) under a nitrogen atmosphere. The reaction mixture was cooled to 0°C then

TBDMSOTf (936 µL, 4.08 mmol, 1.2 eq.) was added dropwise, and it was stirred at 0°C. When the reaction was completed (~2 hours), the mixture was diluted with water and extracted with hexanes three times. The combined organic phases were washed two times with saturated $NaHCO_3$ solution and once with brine, dried over anhydrous Na_2SO_4, filtered and the solvent was removed under reduced pressure. The silyl-enol ether intermediate was obtained as a colorless oil (1.307 g, 94%) and was used in the next step without further purification.
To an anhydrous dimethyl sulfoxide (18 mL) solution of the intermediate were added 4Å molecular sieves, and $Pd(OAc)_2$ (71.8mg, 320.0 mg, 0.1 eq.), and the atmosphere was charged with 3 bars of O_2. The reaction mixture was stirred at 60°C. After completion (~ 18 hours), the reaction mixture was cooled to 25°C, purged with N_2 and 50 mL of diethyl-ether was added and it was stirred for a further 1 hour. The reaction mixture was filtered through a pad of Celite then it was washed with water twice. The aqueous phase was extracted with 30 mL of diethyl-ether three times, then the combined organics were washed three times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered and the solvent was removed under reduced pressure. Purification by flash chromatography on silica gel (hexanes, 0-25% ethyl acetate) to give the product as white solid (179 mg, 38%).

ee: 99+% with catalyst **3a**
ee: -99+% with catalyst **3b**
LC-MS ESI $[M-(^tBu)+H^+]^+$= 237

^1H NMR: (500 MHz, $CDCl_3$): 8.03 (d, J = 1.9 Hz, 1H), 5.39 – 5.33 (m, 1H), 5.14 (d, J = 1.2 Hz, 1H), 3.82 (s, 3H), 3.60 (dt, J = 4.8, 1.0 Hz, 1H), 3.01 – 2.92 (m, 1H), 2.73 – 2.60 (m, 1H), 2.41 – 2.25 (m, 2H), 1.51 (s, 9H)
13C NMR: (125 MHz, $CDCl_3$): 192.0, 163.0, 155.7, 142.0, 131.0, 114.0, 82.2, 58.5, 52.9, 51.8, 42.2, 41.1, 28.2;

1-benzyl 3-methyl-3-methyl-6-oxocyclohex-1-ene-1,3-dicarboxylate: (RU75/RU76)

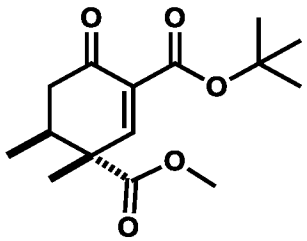


Following the **General Procedure A** using: benzyl 3-oxopent-4-enoate (300 mg, 1.5 mmol), methyl 2-methyl-3-oxopropanoate (190 mg, 1.6 mmol), and triethylamine (41 µl, 0.3 mmol) in 1,4-dioxane (1.5 mL, 1.0 M) at room temperature for 2 days. After removal of the volatile compounds under reduced pressure, purification by flash chromatography on silica gel (hexanes 0-25% ethyl acetate) gave the product as a yellow oil (99 mg, 22%).

ee: 92% with catalyst **3a**
ee: -80% with catalyst **3b**
LC-MS ESI $[M+H^+]^+$= 303

^1H NMR: (500 MHz, $CDCl_3$) 7.51 (s, 1H), 7.43 – 7.39 (m, 2H), 7.36 (ddd, J = 5.9, 3.5, 1.2 Hz, 2H), 7.32 (dt, J = 5.4, 2.1 Hz, 1H), 5.25 (d, J = 3.4 Hz, 2H), 3.74 (s, 3H), 2.61 – 2.51 (m, 2H), 2.51 – 2.43 (m, 1H), 1.98 (ddd, J = 14.0, 9.4, 5.0 Hz, 1H), 1.47 (s, 3H).
^{13}C NMR: (125 MHz, $CDCl_3$) 193.2, 173.5, 164.1, 155.9, 135.5, 131.7, 128.6, 128.6, 128.3, 128.2, 67.1, 52.9, 44.4, 35.3, 32.0, 24.6;

1-(tert-butyl) 3-methyl 3,4-dimethyl-6-oxocyclohex-1-ene-1,3-dicarboxylate: (RU78/RU79)

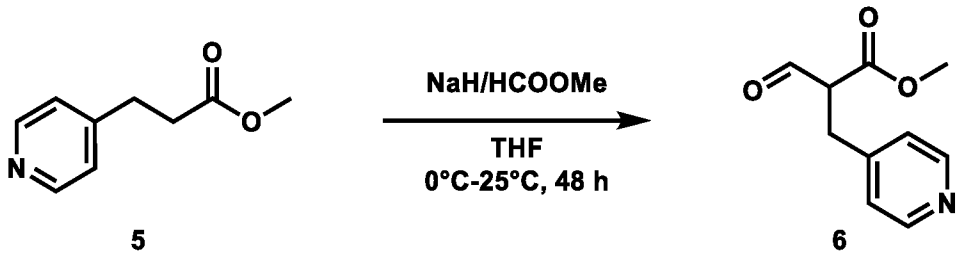


Following the **General Procedure A** using: *tert*-butyl (E)-3-oxohex-4-enoate (184 mg, 1.0 mmol), methyl 2-methyl-3-oxopropanoate (174 mg, 1.5 mmol), and **3a** (27,4 mg, 0.04 mmol) in 1,4-dioxane: hexanes 9:1 (1.0 mL, 1.0 M) at room temperature for 6 days. After removal of the volatile compounds under reduced pressure, purification by flash chromatography on silica gel (hexanes 0-25% ethyl acetate) gave the product as a yellow oil (52 mg, 18%).

dr: >20:1
ee: 89% with catalyst **3a**
ee: -75% with catalyst **3b**
LC-MS ESI $[M-(^tBu)+H^+]^+$= 227

^{1}H NMR: (500 MHz, $CDCl_3$) 7.27 (s, 1H), 3.75 (s, 3H), 2.70 (dqd, J = 11.3, 6.9, 4.4 Hz, 1H), 2.47 (dd, J = 16.8, 4.4 Hz, 1H), 2.30 (dd, J = 16.8, 11.7 Hz, 1H), 1.50 (s, 9H), 1.33 (s, 3H), 0.99 (d, J = 6.9 Hz, 3H).
^{13}C NMR: (125 MHz, $CDCl_3$) 193.6, 174.2, 163.6, 154.8, 132.7, 82.3, 52.9, 48.5, 42.8, 34.7, 28.2, 16.7, 16.2;

Methyl 2-formyl-3-(pyridin-4-yl)propanoate (11)

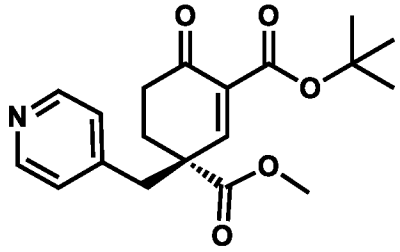


5 6

Compound **5** was synthesized according to literature procedures (Yuan et al., 2012).
A flame-dried vial under a nitrogen atmosphere was charged with NaH (73.0mg, 3.00mmol, 1 eq.) anhydrous THF (9 mL) and methyl-formate (1.90 mL, 30.0 mmol, 10 eq.). The reaction mixture was cooled to 0°C then a THF solution (3 mL) of compound **5** was added dropwise and was stirred for 10 minutes at 0°C. The reaction mixture was warmed to 25°C where it was stirred for 2 days. When the reaction was completed, the reaction was quenched with 50 mL of water and extracted with diethyl ether three times. The pH of the aqueous phase was adjusted to 7 by the addition of 10% citric acid solution then it was extracted three times with ethyl acetate. The combined ethyl acetate phases were dried over anhydrous Na_2SO_4, filtered and the solvent was removed under reduced pressure. Compound **6** was obtained without further purification as a yellow solid (205 mg, 35%).

^{1}H NMR: (500 MHz, $CDCl_3$): 8.47-8.42 (m, 2H), 8.03 (s, 1H), 7.31 (d, J = 5.1 Hz, 2H), 3.69 (s, 3H), 3.68 (s, 1H), 3.40 (s, 2H)

1-(*tert*-butyl) 3-methyl (*S*)-6-oxo-3-(pyridin-4-ylmethyl)cyclohex-1-ene-1,3-dicarboxylate: (RU80)

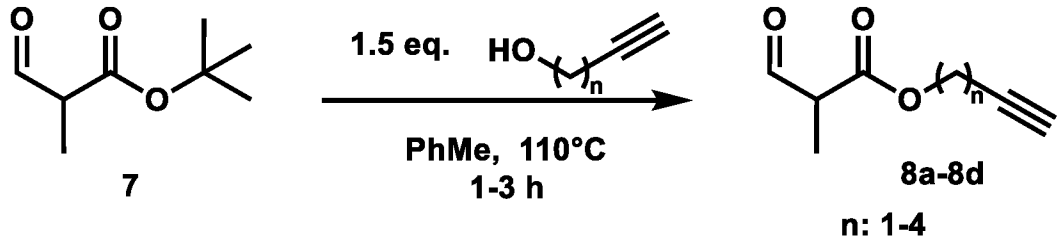


Following the **General Procedure A** using: *tert*-butyl 3-oxopent-4-enoate (201 mg, 1.18 mmol), methyl 2-formyl-3-(pyridin-4-yl)propanoate (209 mg, 1.08 mmol), and **3a** (14,7 mg, 0.02 mmol) in 1,4-dioxane (1.1 mL, 1.0 M) at room temperature for 6 days. After removal of the volatile compounds under reduced pressure, purification by flash chromatography on silica gel (hexanes 0-25% ethyl acetate) gave the product as a yellow oil (120 mg, 32%).

ee: 90% with catalyst **3a**
LC-MS ESI $[M+H^+]^+$= 346

^{1}H NMR: (500 MHz, CDCl3) 8.53 (d, J = 5.3 Hz, 2H), 7.33 (s, J = 11.0 Hz, 1H), 7.04 (t, J = 8.1 Hz, 2H), 3.71 (d, J = 5.4 Hz, 3H), 3.08 (d, J = 13.1 Hz, 2H), 2.59 – 2.46 (m, 2H), 2.43 – 2.35 (m, 1H), 2.03 (dq, J = 9.3, 5.7 Hz, 1H), 1.50 (s, 9H).
^{13}C NMR: (125 MHz, CDCl3) 193.0, 172.1, 163.4, 151.5, 150.1, 144.1, 134.2, 125.1, 82.5, 52.9, 48.9, 43.6, 35.2, 30.3, 28.1;

General procedure B for the transesterification of compound 6



Compound **7** was synthesized according to literature procedures (El-Mansy et al., 2016).

Compound 7 (1.00 g, 6.321 mmol, 1 eq.) was dissolved in toluene (30 mL) then the appropriate propargyl alcohol derivative (1.5 eq.) was added, and the mixture was heated to 110°C where it was kept for until full conversion was achieved (1-3 hours). The mixture was cooled to room temperature and then concentrated under reduced pressure at 30°C. Compound **8a-8d** were obtained by vacuum distillation as colorless oils.

n=1; Prop-2-yn-1-yl 2-methyl-3-oxopropanoate: (624.0 mg, 68%)

^{1}H-NMR (500MHz, $CDCl_3$) enol-form: 11.04 (d, J = 12.8 Hz, 1H), 7.03 (dq, J = 12.8, 1.2 Hz, 1H), 4.79 (d, J = 2.5 Hz, 2H), 2.50 (t, J = 2.5 Hz, 1H), 1.72 (d, J = 1.2 Hz, 3H).
^{13}C-NMR (125 MHz, $CDCl_3$) enol form: 197.3, 169.9, 83.7, 69.0, 62.4, 46.1, 18.1;

n=2; But-3-yn-1-yl 2-methyl-3-oxopropanoate: (444.0 mg, 46%)

^{1}H-NMR (500MHz, $CDCl_3$) enol form: 11.14 (d, J = 12.6 Hz, 1H), 7.01 (dd, J = 12.6, 1.2 Hz, 1H), 4.30 (t, J = 6.8 Hz, 2H), 2.58 (dtd, J = 7.3, 6.8, 2.6 Hz, 2H), 2.01 (td, J = 2.7, 1.5 Hz, 1H), 1.70 (d, J = 1.2 Hz, 3H).
^{13}C-NMR (75 MHz, $CDCl_3$) enol form: 196.7, 169.2, 77.0, 75.7, 52.9, 51.9, 28.1, 10.3;

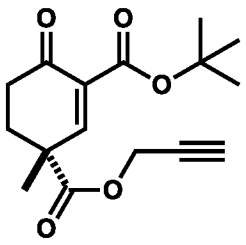
n=3; Pent-4-yn-1-yl 2-methyl-3-oxopropanoate: (574.0 mg, 54%)

^{1}H-NMR (300MHz, $CDCl_3$) enol form: 11.25 (d, J = 12.5 Hz, 1H), 7.00 (dd, J = 12.5, 1.2 Hz, 1H), 4.29 (t, J = 6.2 Hz, 2H), 2.34 – 2.29 (m, 2H), 1.97 (dt, J = 5.0, 2.7 Hz, 1H), 1.95 – 1.88 (m, 2H), 1.68 (d, J = 1.2 Hz, 3H).
^{13}C-NMR (75 MHz, $CDCl_3$) enol form: 196.9, 160.5, 79.6, 70.3, 62.9, 52.6, 28.0, 18.9, 12.3;

n=4; Hex-5-yn-1-yl 2-methyl-3-oxopropanoat): (641.0 mg, 56%)

^{1}H-NMR (300MHz, $CDCl_3$) enol form: 11.28 (d, J = 12.5 Hz, 1H), 7.00 (dq, J = 12.5, 1.2 Hz, 1H), 4.22 (t, J = 6.41 Hz, 2H), 2.30 – 2.19 (m, 2H), 1.99 – 1.92 (m, 1H), 1.88 – 1.74 (m, 2H), 1.68 (d, J = 1.2 Hz, 3H), 1.66-1.59 (m, 2H);
^{13}C-NMR (75 MHz, $CDCl_3$) enol form: 197.2, 162.8, 89.2, 69.4, 61.5, 52.6, 31.0, 27.1, 21.4, 15.0;

1-(tert-butyl) 3-(prop-2-yn-1-yl) 3-methyl-6-oxocyclohex-1-ene-1,3-dicarboxylate: (RU83/RU188)



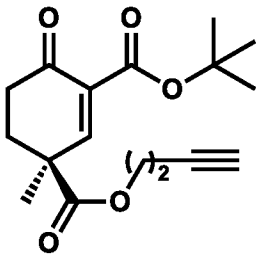
Following the **General Procedure A** using: *tert*-butyl 3-oxopent-4-enoate (300.0 mg, 1.76 mmol), prop-2-yn-1-yl 2-methyl-3-oxopropanoate (271.7 mg, 1.94 mmol) and catalyst **3a/3b** (24.14 mg, 0.0353 mmol) in 1,4-dioxane (1.8 mL, 1.0 M) at room temperature for 3 days. After removal of the volatile compounds under reduced pressure, purification by flash chromatography on silica gel (hexanes 0-25% ethyl acetate) gave the product as a colorless oil (433.0 mg, 84%).

ee: 92% with catalyst **3a**
ee: -87% with catalyst **3b**

LC-MS ESI [M-(^{t}Bu)+H$^+$]$^+$= 237

^{1}H-NMR (300 MHz, $CDCl_3$): 7.35 (s,1H), 4.74 (dd, 2H), 2.58-2.53 (m, 2H), 2.50 (s, 1H), 2.48–2.43 (m, 1H) 2.04–1.96 (m, 1H), 1.52 (s, 9H), 1.50 (s, 3H)
^{13}C-NMR (75 MHz, $CDCl_3$): 199.5, 172.7, 163.7, 153.3, 133.7, 82.4, 75.6, 53.2, 44.2, 35.3, 32.0, 28.5, 28.2, 24.5;

3-(but-3-yn-1-yl) 1-(tert-butyl) (*S*)-3-methyl-6-oxocyclohex-1-ene-1,3-dicarboxylate (RU100)

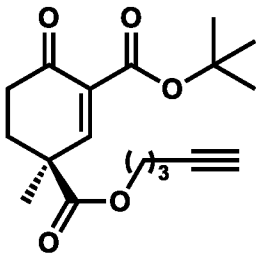


Following the **General Procedure A** using: *tert*-butyl 3-oxopent-4-enoate (400.0 mg, 2.35 mmol), but-3-yn-1-yl 2-methyl-3-oxopropanoate (400.0 mg, 2.59 mmol) and catalyst **3b** (24.57 mg, 0.047 mmol) in 1,4-dioxane (2.35 mL, 1.0 M) at room temperature for 3 days. After removal of the volatile compounds under reduced pressure, purification by flash chromatography on silica gel (hexanes 0-25% ethyl acetate) gave the product as a colorless oil (394.0 mg, 55%).

ee: -84% with catalyst **3b**
LC-MS ESI [M-(^{t}Bu)+H$^+$]$^+$= 251

^{1}H-NMR (300 MHz, $CDCl_3$): 7.34 (s, 1H), 4.26 (m, 2H), 2.55(m, 2H), 2.51 (m, 1H), 2.47(s, 1H), 2.44 (m, 1H), 1.98 (m, 2H), 1.50 (s, 9H), 1.49 (s, 3H)
^{13}C-NMR (75 MHz, $CDCl_3$): 193.5, 173.0, 163.6, 153.7, 133.4, 82.1, 79.4, 70.2, 63.0, 44.2, 35.3, 31.9, 28.1, 24.6, 18.9;

1-(tert-butyl) 3-(pent-4-yn-1-yl) (*S*)-3-methyl-6-oxocyclohex-1-ene-1,3-dicarboxylate (RU101)

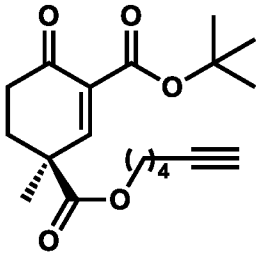


Following the **General Procedure A** using: *tert*-butyl 3-oxopent-4-enoate (370.0 mg, 2.17 mmol), pent-4-yn-1-yl 2-methyl-3-oxopropanoate (402.0 mg, 2.39 mmol) and catalyst **3b** (22.7 mg, 0.043 mmol) in 1,4-dioxane (2.10 mL, 1.0 M) at room temperature for 3 days. After removal of the volatile compounds under reduced pressure, purification by flash chromatography on silica gel (hexanes 0-25% ethyl acetate) gave the product as a colorless oil (354.0 mg, 51%).

ee: -81% with catalyst **3b**
LC-MS ESI [M-(^{t}Bu)+H$^+$]$^+$= 265

^{1}H-NMR (300 MHz, $CDCl_3$): 7.35 (s, 1H), 4.27 (t, 2H), 2.57-2.51 (m, 2H), 2.47-2.43 (m, 1H), 2.29 (td, 2H), 2.03-2.00 (m, 1H), 1.99-1.97 (m, 1H), 1.91-1.86 (m, 2H), 1.52 (s, 9H), 1.49 (s, 3H)
^{13}C-NMR (75 MHz, $CDCl_3$): 193.4, 173.2, 163.9, 153.7, 133.3, 82.5, 82.1, 69.4, 64.2, 44.2, 35.3, 32.0, 28.1, 27.3, 24.5, 15.1;

1-(tert-butyl) 3-(hex-5-yn-1-yl) (*S*)-3-methyl-6-oxocyclohex-1-ene-1,3-dicarboxylate (RU102)

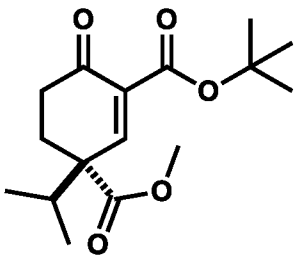


Following the **General Procedure A** using: *tert*-butyl 3-oxopent-4-enoate (510.0 mg, 3.00 mmol), hex-5-yn-1-yl 2-methyl-3-oxopropanoate (600.6 mg, 3.30 mmol) and catalyst **3b** (31.3 mg, 0.020 mmol) in 1,4-dioxane (3.00 mL, 1.0 M) at room temperature for 3 days. After removal of the volatile compounds under reduced pressure, purification by flash chromatography on silica gel (hexanes 0-25% ethyl acetate) gave the product as a colorless oil (359.0 mg, 36%).

ee: -86% with catalyst **3b**
LC-MS ESI $[M-(^tBu)+H^+]^+$= 279

^{1}H-NMR (300 MHz, $CDCl_3$): 7.35 (s, 1H), 4.18 (dd, 2H), 2.54-2.51 (m, 2H), 2.47-2.42 (m, 1H), 2.23 (td, 2H), 2.03-1.98 (m, 1H), 1.96 (s, 1H), 1.78 (dd, 2H), 1.61-1.56 (m, 2H), 1.51 (s, 9H), 1.48 (s, 3H)
^{13}C-NMR (75 MHz, $CDCl_3$): 193.7, 173.4, 163.7, 154.1, 133.3, 82.3, 69.1, 63.4, 44.4, 35.5, 32.1, 28.5, 28.2, 27.7, 25.0, 24.7, 18.2;

1-(*tert*-butyl) 3-methyl 3-isopropyl-6-oxocyclohex-1-ene-1,3-dicarboxylate (RU217/218)



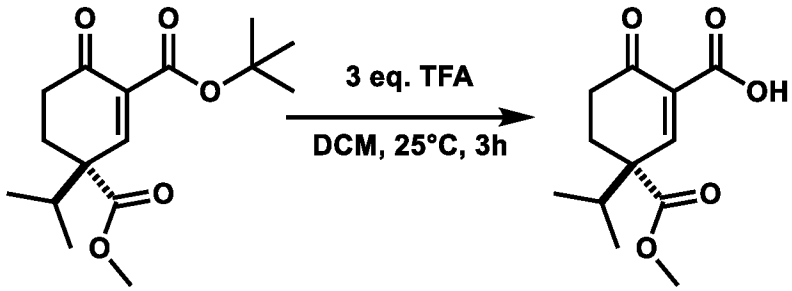
Following the **General Procedure A** using: *tert*-butyl 3-oxopent-4-enoate (1.00 g, 5.88 mmol), methyl 2-formyl-3-methylbutanoate (931.7 mg, 6.46 mmol) and catalyst **3a/3b** (80.5 mg, 0.118 mmol) in 1,4-dioxane (5.9 mL, 1.0 M) at room temperature for 3 days. After removal of the volatile compounds under reduced pressure, purification by flash chromatography on silica gel (hexanes 0-25% ethyl acetate) gave the product as a colorless oil (1.442 g, 83%).

ee: 99+% with catalyst **3a**
ee: -99+% with catalyst **3b**

LC-MS ESI $[M-(^tBu)+H^+]^+$= 241

^{1}H-NMR (300 MHz, $CDCl_3$): 7.29 (d, J = 1.9 Hz, 1H), 3.72 (s, 3H), 2.57 – 2.41 (m, 2H), 2.35 (dddd, J = 13.6, 5.0, 4.0, 1.9 Hz, 1H), 2.23 (hept, J = 6.9 Hz, 1H), 1.95 (ddd, J = 13.7, 12.7, 5.1 Hz, 1H), 1.49 (s, 9H), 0.92 (dd, J = 13.1, 6.9 Hz, 6H).
^{13}C-NMR (75 MHz, $CDCl_3$): 194.2, 172.7, 163.8, 154.0, 134.1, 52.6, 52.1, 36.0, 35.8, 28.2, 25.5, 17.8, 17.6.

3-isopropyl-3-(methoxycarbonyl)-6-oxocyclohex-1-ene-1-carboxylic acid (9)

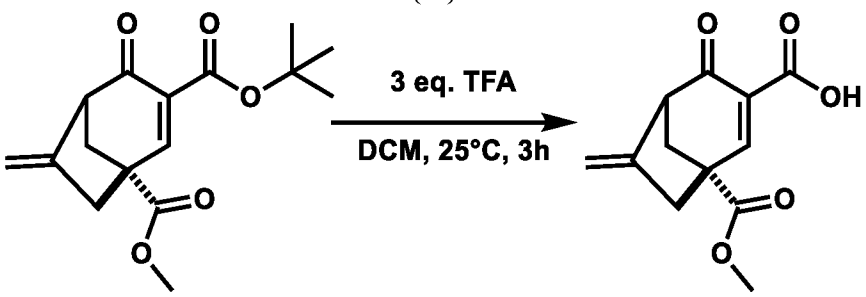


Trifluoroacetic acid (312 µL, 4.05 mmol, 3 eq.) was added to a solution of compound **RU217/218** (400.0 mg, 1.35 mmol, 1 eq.) in methylene chloride (10.4 mL) and then the mixture was stirred at 25°C. The reaction was monitored by TLC and when it was completed (~3 hours) the reaction mixture was evaporated. The remaining trifluoroacetic acid was removed by redissolving the mixture in toluene and evaporating to dryness at 50°C three times. Compound **9** was obtained as a yellow oil in quantitative yield (324.0 mg) and was used in the next step without further purification.

LC-MS ESI $[M+H^+]^+$= 241;

^{1}H NMR (500 MHz, $CDCl_3$): 8.27 (d, J = 1.9 Hz, 1H), 3.76 (s, 3H), 2.73 – 2.64 (m, 2H), 2.51 – 2.41 (m, 1H), 2.35 (h, J = 6.9 Hz, 1H), 2.04 (ddd, J = 13.8, 10.5, 7.7 Hz, 1H), 0.98 (dd, J = 12.8, 6.9 Hz, 6H).
^{13}C NMR (125 MHz, $CDCl_3$): 202.6, 171.5, 165.4, 163.6, 53.4, 53.0, 36.1, 35.0, 25.4, 18.0, 17.6.

1-(methoxycarbonyl)-6-methylene-4-oxobicyclo[3.2.1]oct-2-ene-3-carboxylic acid (10)

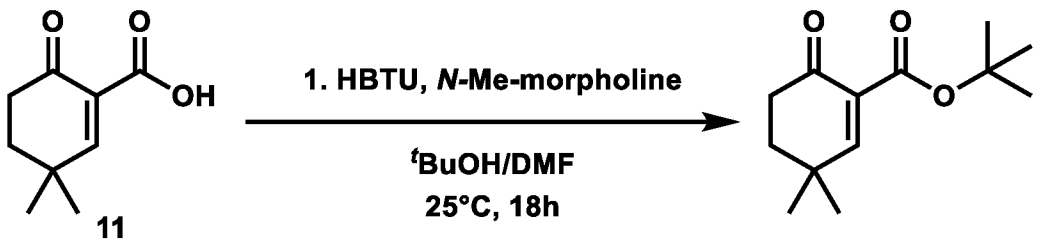


Trifluoroacetic acid (158 µL, 2.05 mmol, 3 eq.) was added to a solution of compound **RU73/77** (200.0 mg, 0.684 mmol, 1 eq.) in methylene chloride (4.0 mL) and then the mixture was stirred at 25°C. The reaction was monitored by TLC and when it was completed (~3 hours) the reaction mixture was evaporated. The remaining trifluoroacetic acid was removed by redissolving the mixture in toluene and evaporating to dryness at 50°C three times. Compound **10** was obtained as a yellow oil in quantitative yield (160.0 mg) and was used in the next step without further purification.

LC-MS ESI $[M+H^+]^+$= 237;

^{1}H NMR (500 MHz, $CDCl_3$): 8.80 (d, J = 2.0 Hz, 1H), 5.45 (t, J = 1.7 Hz, 1H), 5.29 – 5.24 (m, 1H), 3.85 (s, 3H), 3.76 (d, J = 4.9 Hz, 1H), 3.07 (dt, J = 16.1, 2.6 Hz, 1H), 2.68 – 2.62 (m, 1H), 2.45 (ddd, J = 11.8, 4.9, 2.1 Hz, 1H), 2.36 (dd, J = 11.9, 2.5 Hz, 1H).
^{13}C NMR (125 MHz, $CDCl_3$): 199.6, 171.3, 164.5, 163.1, 140.4, 124.8, 116.0, 57.0, 53.1, 52.6, 42.5, 40.1.

***Tert*-butyl 3,3-dimethyl-6-oxocyclohex-1-ene-1-carboxylate: (RU146)**



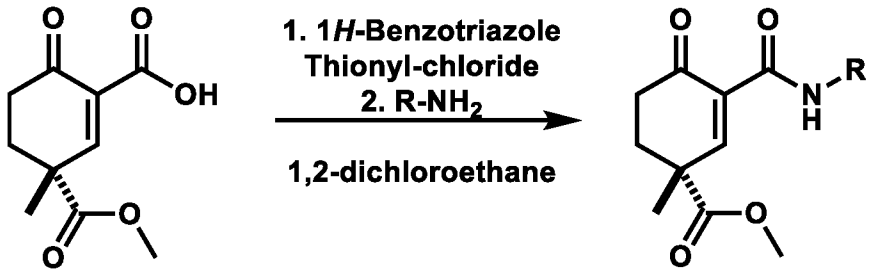
Compound **11** was prepared according to the literature procedures (Flaugh et al., 1980) (Deny et al., 2016).

Compound **11** (100.0 mg, 0.595 mmol, 1 eq.) was dissolved in anhydrous dimethylformamide (5.5 mL) in a flame-dried vial under a nitrogen atmosphere, HBTU (225.5 mg, 0.595 mmol, 1.5 eq.) and *N*-methylmorpholine (131 µL, 0.825 mmol, 2.0 eq.) were added and the mixture was stirred at 25°C. After 30 minutes *tert*-butyl alcohol (114 µL, 0.275 mmol, 2 eq.) was added and the reaction mixture was stirred at 25°C. The reaction was monitored by HPLC-MS and when the starting material was consumed (~3 days) the reaction mixture was diluted with ethyl acetate, washed two times with a 5% citric acid solution, two times with a saturated aqueous Na_2CO_3 solution, and two times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The resulting crude product was purified by preparative RP-HPLC using a gradient method on a Gemini® 5µm C18 110 Å column (H_2O:MeCN = 95:5 (0.1 % HCOOH) to 100% MeCN (0.1 % HCOOH)). Compound **RU146** was obtained as a yellow solid (19.4 mg, 14.5%).

LC-MS ESI $[M-(^tBu)+H^+]^+$= 169

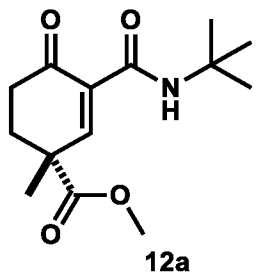
^{1}H-NMR (500 MHz, $CDCl_3$): 7.17 (t, J = 0.9 Hz, 1H), 2.51 (t, J = 6.8 Hz, 2H), 1.88 (td, J = 6.8, 1.0 Hz, 2H), 1.52 (s, 9H), 1.22 (s, 6H).
^{13}C-NMR (125 MHz, $CDCl_3$): 194.7, 164.3, 162.4, 131.8, 81.8, 35.7, 35.4, 33.4, 28.2, 27.6;

General procedure C for the amidation of compound RU57:



12a-g

1*H*-benzotriazole (4 eq.) was dissolved in 1,2-dichloroethane (0.75 M) under a nitrogen atmosphere and was left to stir for 10 minutes at 25°C. To this solution thionyl chloride (1.1 eq.) was added dropwise and was stirred for 30 minutes at 25°C. The reaction mixture was cooled to 0°C and was stirred for 15 minutes. A 1,2-dichloroethane solution of compound **RU57** (0.88 M, 1 eq.) was added dropwise to the reaction mixture which was stirred at 0°C for 24 hours. When the carboxylic acid was completely consumed the reaction mixture was filtered, the filtrate was washed two times with 1,2-dichloroethane, and the organic phase was washed three times with a saturated $NaHCO_3$ solution. The organic phase was dried over anhydrous Na_2SO_4, filtered, then the corresponding amine/amine hydrochloride and triethylamine (1.2 and 2.4 eq. respectively) was added, and the reaction mixture was stirred for 1 hour. After the reaction was completed, the mixture was washed two times with 10% HCl solution, two times with a saturated Na_2CO_3 solution, and once with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, the solvent was removed under reduced pressure. The product was purified with flash chromatography (hexanes 0-25% ethyl acetate) if necessary.

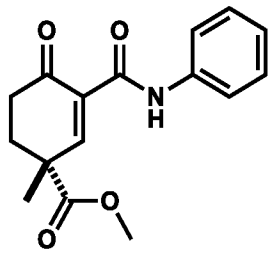


12a

Compound **12a** (**RU58**) was synthesized according to the **General procedure C** using: 1*H*-benzotriazole (560 mg, 4.72 mmol, 4 eq.), thionyl chloride (94.0 µL, 1.30 mmol, 1.1 eq.) compound **RU57** (250.0 mg, 1.18 mmol, 1 eq.) *tert*-butylamine (149.0 µL, 1.41 mmol, 1.2 eq.) and was obtained as a yellow oil (177mg, 56%).

LC-MS ESI $[M+H^+]^+$= 268

1H NMR: (500 MHz, $CDCl_3$): 8.57 (s, 1H), 8.07 (s, 1H), 3.71 (s, 3H), 2.64 – 2.51 (m, 2H), 2.48 – 2.41 (m, 1H), 1.92 (ddd, J = 13.8, 10.1, 4.6 Hz, 1H), 1.47 (s, 3H), 1.37 (s, 9H).
13C NMR: (125 MHz, $CDCl_3$) 199.4, 173.7, 161.4, 159.6, 130.0, 52.9, 51.2, 44.9, 36.1, 32.2, 28.8, 24.8.

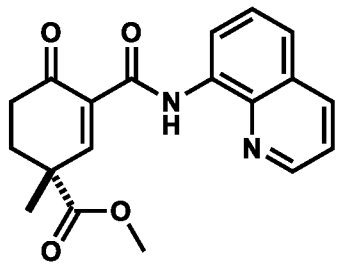


12b

Compound **12b** (**RU59**) was synthesized according to the **General procedure C** using: 1*H*-benzotriazole (560 mg, 4.72 mmol, 4 eq.), thionyl chloride (94.0 µL, 1.30 mmol, 1.1 eq.) compound **RU57** (250.0 mg, 1.18 mmol, 1 eq.) aniline (129.0 µL, 1.41 mmol, 1.2 eq.) and was obtained as an orange oil (267 mg, 79%).

LC-MS ESI $[M+H^+]^+$= 288

1H NMR: (500 MHz, $CDCl_3$) 10.68 (s, 1H), 8.25 (s, 1H), 7.67 – 7.62 (m, 2H), 7.35 – 7.30 (m, 2H), 7.14 – 7.08 (m, 1H), 3.76 (s, 3H), 2.73 – 2.60 (m, 2H), 2.56 – 2.49 (m, 1H), 2.05 – 1.97 (m, 1H), 1.54 (s, 3H).
13C NMR: (125 MHz, $CDCl_3$) 199.6, 173.5, 161.5, 160.4, 138.0, 129.3, 129.1, 124.6, 120.6, 53.0, 45.2, 36.0, 32.2, 24.8;

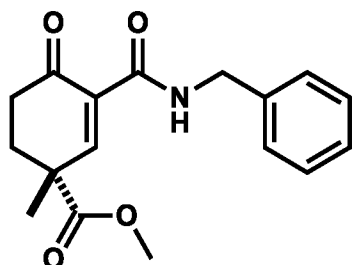


12c

Compound **12c** (**RU65**) was synthesized according to the **General procedure C** using: 1*H*-benzotriazole (532 mg, 4.47 mmol, 4 eq.), thionyl chloride (89.0 µL, 0.15 mmol, 1.1 eq.) compound **RU57** (237.0 mg, 1.12 mmol, 1 eq.) quinoline-8-amine (193 mg, 1.34 mmol, 1.2 eq.) and was obtained as a brown oil (237 mg, 63%).

LC-MS ESI $[M+H^+]^+$= 339

^{1}H NMR: (500 MHz, CDCl3) 12.50 (s, 1H), 8.96 – 8.93 (m, 1H), 8.87 (dd, J = 6.5, 2.5 Hz, 1H), 8.26 (s, 1H), 8.11 (dd, J = 8.3, 1.7 Hz, 1H), 7.54 – 7.48 (m, 2H), 7.42 (dd, J = 8.2, 4.2 Hz, 1H), 3.74 (s, 3H), 2.78 – 2.64 (m, 2H), 2.57 – 2.49 (m, 1H), 2.06 – 1.98 (m, 1H), 1.54 (s, 3H).
^{13}C NMR: (125 MHz, CDCl3) 198.7, 173.5, 160.8, 160.7, 148.8, 139.5, 136.1, 135.2, 130.1, 128.1, 127.2, 122.3, 121.6, 118.0, 52.9, 45.1, 35.9, 32.1, 24.7;

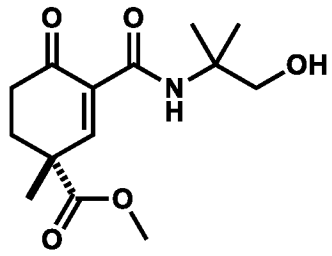


12d

Compound **12d** (**RU66**) was synthesized according to the **General procedure C** using: 1*H*-benzotriazole (337 mg, 2.83 mmol, 4 eq.), thionyl chloride (56.5 µL, 0.78 mmol, 1.1 eq.) compound **RU57** (150.0 mg, 0.71 mmol, 1 eq.) benzylamine (92.0 µL, 0.85 mmol, 1.2 eq.) and was obtained as a yellow oil (168 mg, 79%).

LC-MS ESI $[M+H^+]^+$= 302

^{1}H NMR: (500 MHz, $CDCl_3$): 8.99 (s, 1H), 8.18 (s, 1H), 7.33-7.32 (m, 4H), 4.56 (dd, J = 5.8, 2.5 Hz, 2H), 3.75 (s, 3H), 2.69 – 2.55 (m, 2H), 2.55 – 2.45 (m, 1H), 1.97 (ddd, J = 13.7, 10.1, 4.7 Hz, 1H), 1.52 (s, 3H)
^{13}C NMR: (125 MHz, $CDCl_3$): 199.2, 173.6, 162.6, 160.7, 138.3, 129.1, 128.7, 127.8, 127.4, 53.0, 45.0, 43.7, 36.0, 32.2, 24.8;

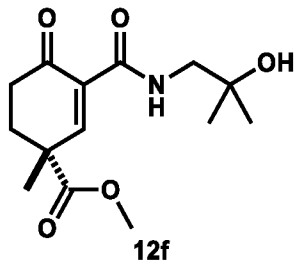


12e

Compound **12e** (**RU84**) was synthesized according to the **General procedure C** using: 1*H*-benzotriazole (674 mg, 5.66 mmol, 4 eq.), thionyl chloride (113.0 µL, 1.56 mmol, 1.1 eq.) compound **RU57** (300.0 mg, 1.41 mmol, 1 eq.) 2-amino-2-methyl-1-propanol (168.0 mg, 1.70 mmol, 1.2 eq.) and was obtained as a yellow oil in 55%.

LC-MS ESI $[M+H^+]^+$= 284

^{1}H NMR (300MHz, $CDCl_3$): 8.96 (s, 1H), 8.13 (s, 1H), 3.76 (s, 3H), 3.62 (d, J = 4.1 Hz, 2H), 2.65 – 2.55 (m, 2H), 2.54 – 2.41 (m, 1H), 2.05 – 1.90 (m, 1H), 1.51 (s, 3H), 1.34 (s, 6H).
^{13}C (75MHz, $CDCl_3$): 199.3; 173.4; 162.9; 160.7; 129.1; 70.8; 56.4; 53.0; 45.0; 35.9; 32.1; 29.8; 24.8; 24.8; 24.7;

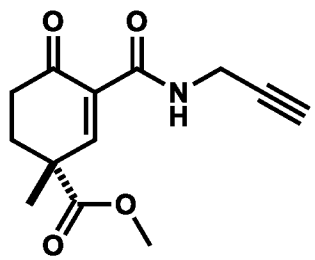


12f

Compound **12f** (**RU85**) was synthesized according to the **General procedure C** using: 1*H*-benzotriazole (1.15g, 9.69 mmol, 4 eq.), thionyl chloride (194.5 µL, 2.66 mmol, 1.1 eq.) compound **RU57** (514.0 mg, 2.42 mmol, 1 eq.) 1-amino-2-methyl-propan-2-ol (259.1 mg, 2.91 mmol, 1.2 eq.) and was obtained as a yellow oil (386.0 mg, 56%).

LC-MS ESI $[M+H^+]^+$= 282

^{1}H NMR (300MHz, $CDCl_3$): 9.00 (s, 1H), 8.15 (s, 1H), 3.75 (s, 3H), 3.39 (dd, J = 5.9, 2.6 Hz, 2H), 2.66-2.57 (m, 2H), 2.50 – 2.44 (m, 1H), 2.04 – 1.92 (m, 1H), 1.52 (s, 3H), 1.25 (s, 6H).
^{13}C (75MHz, $CDCl_3$): 199.1; 173.5; 163.9; 160.7; 128.9; 71.1; 52.9; 50.9; 45.0; 43.5; 35.9; 32.1; 27.5; 27.4; 24.7;



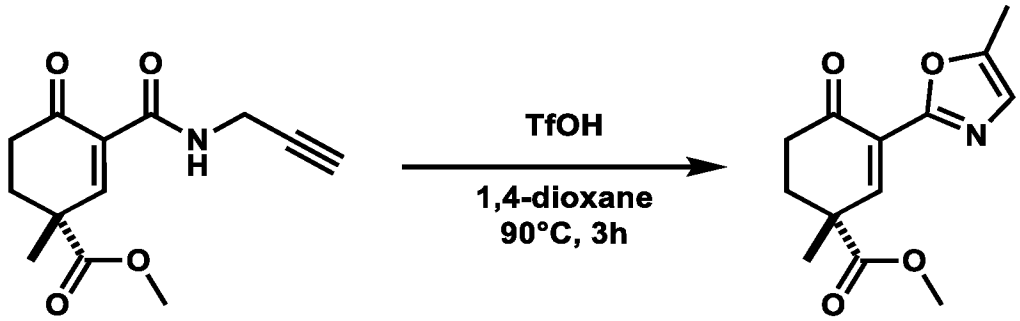
12g

Compound **12g** was synthesized according to the **General procedure C** using: 1*H*-benzotriazole (1.12 g, 9.43 mmol, 4 eq.), thionyl chloride (189 µL, 2.60 mmol, 1.1 eq.) compound **RU57** (500.0 mg, 2.36 mmol, 1 eq.) propargylamine (181 µL, 1.34 mmol, 1.2 eq.) and was obtained as a red oil (182.0 mg, 30%).

LC-MS ESI $[M+H^+]^+$= 250

^{1}H NMR: (500 MHz, $CDCl_3$): 8.88 (s, 1H), 8.15 (s, 1H), 4.16-4.13 (m, 2H), 3.75 (s, 3H), 2.88 (s, 1H), 2.63-2.57 (m, 2H), 2.52-2.46 (m, 1H), 2.24-2.22 (m, 1H), 1.51 (s, 3H)
^{13}C NMR: (125 MHz, $CDCl_3$): 198.9, 173.4, 162.4, 161.0, 128.7, 71.4, 53.0, 44.9, 38.7, 35.8, 32.1, 29.1, 24.7.

Methyl 1-methyl-3-(5-methyloxazol-2-yl)-4-oxocyclohex-2-ene-1-carboxylate: (RU81)

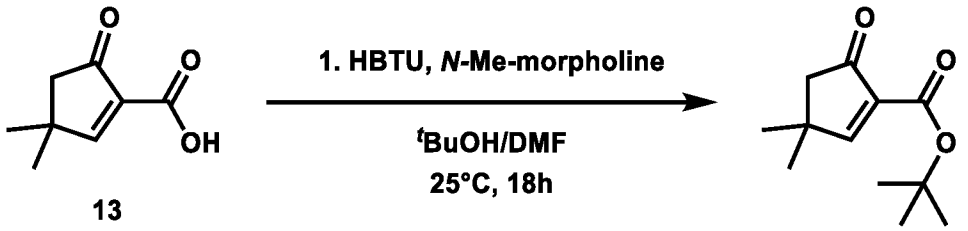


Compound **12g** (75.0 mg, 0.301mmol, 1 eq.) was dissolved in 1,4-dioxane (0.50 mL), TfOH was added (80 µL, 0.903 mmol, 1.1 eq.) and the reaction mixture was stirred at 90°C for 3 hours. After the reaction was completed, the mixture was diluted with ethyl acetate then washed two times with a saturated $NaHCO_3$ solution. The organic phase was washed with brine then dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure. The crude product was purified by column chromatography (hexane: ethyl acetate) and compound **RU81** was obtained as a yellow oil (26.0 mg, 35%).

LC-MS ESI $[M+H^+]^+$= 250

^{1}H NMR (500MHz, $CDCl_3$): 7.53 (s, 1H), 6.79 (d, 1H), 3.75 (s, 3H), 2.68-2.60 (m, 2H), 2.54-2.49 (m, 1H), 2.34 (d, 3H), 2.05-2.00 (m, 1H), 1.51 (s, 3H)
^{13}C (125MHz, $CDCl_3$): 193.7; 173.9; 156.4; 152.0; 149.4; 127.2; 124.0; 52.7; 44.5; 35.5; 32.1; 24.9; 10.9.

Tert-butyl 3,3-dimethyl-5-oxocyclopent-1-ene-1-carboxylate (RU151)



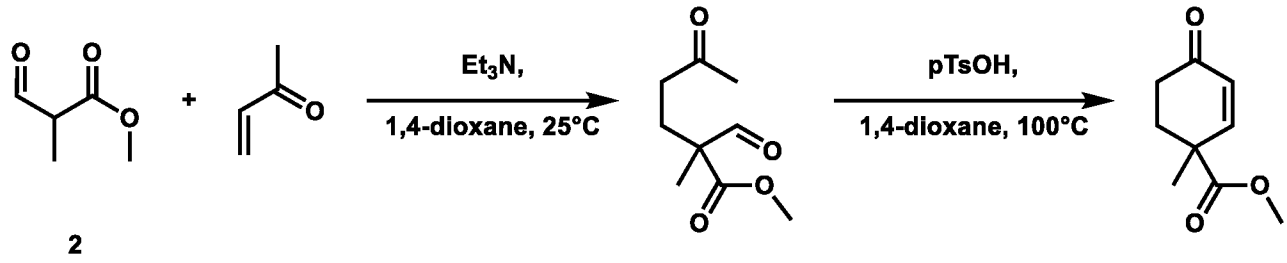
13

Compound **13** was prepared according to the literature procedures (Schultz et al. 1984) (Peelen et al., 2004) (Meyer et al. 2000).

Compound **13** (50.0 mg, 0.324 mmol, 1 eq.) was dissolved in anhydrous dimethylformamide (2.50 mL) in a flame-dried vial under a nitrogen atmosphere, HBTU (123.0 mg, 0.324 mmol, 1 eq.) and *N*-methylmorpholine (71.3 µL, 0.649 mmol, 2.0 eq.) were added and the mixture was stirred at 25°C. After 30 minutes *tert*-butyl alcohol (62.0 µL, 0.649 mmol, 2 eq.) was added and the reaction mixture was stirred at 25°C. The reaction was monitored by HPLC-MS and when the starting material was consumed (~3 days) the reaction mixture was diluted with ethyl acetate, washed two times with a 5% citric acid solution, two times with a saturated aqueous Na_2CO_3 solution, and two times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The resulting crude product was purified by preparative RP-HPLC using a gradient method on a Gemini® 5µm C18 110 Å column (H_2O:MeCN = 95:5 (0.1 % HCOOH) to 100% MeCN (0.1 % HCOOH)). Compound **RU151** was obtained as a yellow oil (40.0 mg, 58.7%).

LC-MS ESI $[M-(^tBu)+H^+]^+$= 155
^{1}H NMR (500MHz, $CDCl_3$): 7.97 (s, 1H), 2.39 (s, 2H), 1.53 (s, 9H), 1.27 (s, 6H).
^{13}C (125MHz, $CDCl_3$): 202.9, 178.7, 161.2, 135.1, 81.9, 51.4, 38.6, 28.2, 27.6;

Methyl 1-methyl-4-oxocyclohex-2-enecarboxylate: (RU56)

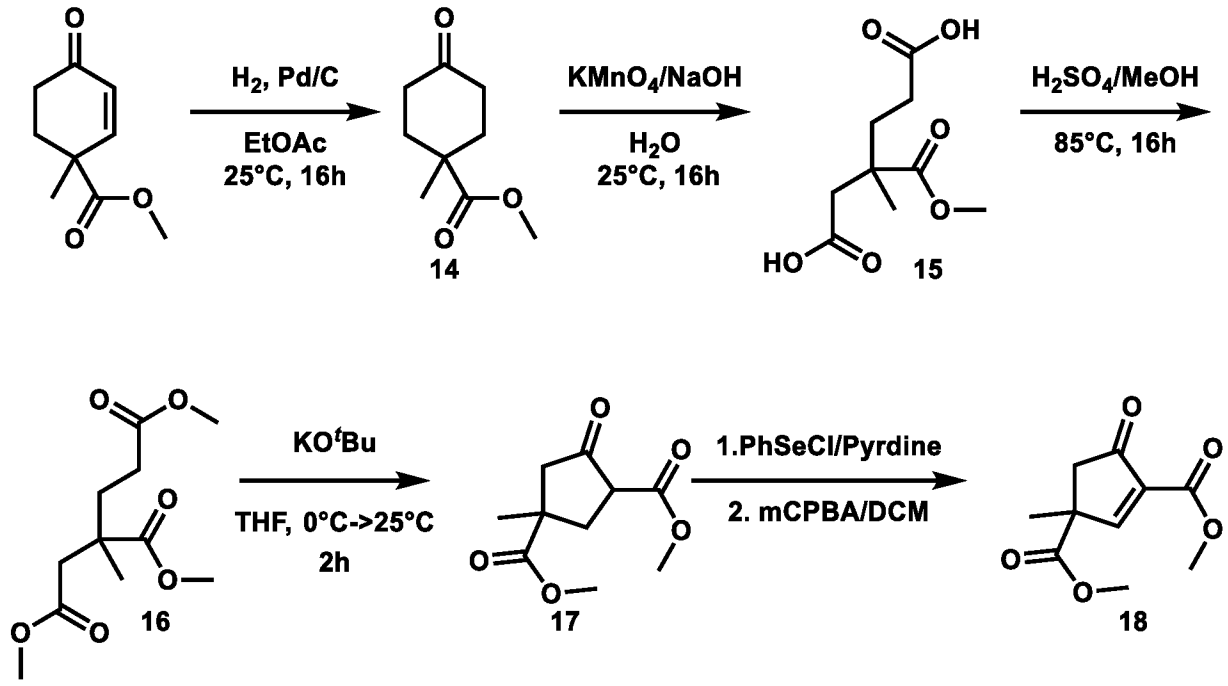


To methyl vinyl ketone (500 µL, 6.00 mmol, 1 eq.), dissolved in 6 mL of 1,4-dioxane, was added 2-methyl-3-oxopropanoate (770.0 mg, 6.60 mmol, 1.1 eq.) and triethylamine (170 µL, 1.5 mmol, 0.25 eq.). The reaction mixture was stirred at room temperature and monitored by TLC. Upon completion, p-toluenesulfonic acid (630 mg, 3.3 mmol, 0.55 eq.) along with 3 mL of 1,4-dioxane was added, and the reaction was stirred at 100°C until complete conversion of the intermediate product. After removal of the solvent under reduced pressure, the residue was purified by flash column chromatography on silica gel (hexanes 0-25% ethyl acetate) to afford compound **RU56** as a yellow oil (131 mg, 51%).

LC-MS ESI $[M+H^+]^+$= 169;

^{1}H NMR: (500 MHz, $CDCl_3$) 6.81 (d, *J* = 10.2 Hz, 1H), 5.89 (d, *J* = 10.2 Hz, 1H), 3.67 (s, 3H), 2.50 – 2.33 (m, 3H), 1.95 – 1.85 (m, 1H), 1.37 (s, 3H).
^{13}C NMR: (125 MHz, $CDCl_3$) 198.1, 174.4, 151.5, 128.6, 52.5, 43.7, 34.4, 32.4, 24.7.

Dimethyl 3-methyl-5-oxocyclopent-1-ene-1,3-dicarboxylate: (18)



Compound **RU56** (517 mg, 3.07 mmol, 1 eq.) was dissolved in ethyl acetate (3.11 mL) in a vial under a nitrogen atmosphere. 20.0 mg of 10% palladium on carbon was added and the reaction mixture was purged with hydrogen with a use of a balloon. After purging the atmosphere, three hydrogen balloons were attached to the vial and the mixture was vigorously stirred for 16 hours at 25°C. The reaction was monitored by HPLC-MS and when the starting material was consumed the reaction mixture was diluted with ethyl acetate and filtered through a pad of Celite which was washed two times with ethyl acetate. The solvent was evaporated under reduced pressure affording compound **14** as a colorless oil without further purification (463 mg, 88%).

LC-MS ESI $[M+H^+]^+$= 171;

^{1}H NMR (500 MHz, $CDCl_3$): 3.76 (s, 3H), 2.47 – 2.37 (m, 4H), 2.36 – 2.27 (m, 2H), 1.75 – 1.63 (m, 2H), 1.31 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$): 210.9, 176.8, 52.2, 42.5, 38.5, 35.3, 25.7.

To a suspension of compound **14** (400.0 mg, 2.35 mmol, 1 eq.) and $KMnO_4$ (742.8 mg, 4.70 mmol, 2 eq.) in water (14 mL) was added a solution of NaOH (35.0 mg, 0.869 mmol, 0.36 eq.) in water (2 mL). The reaction was stirred at 25°C for 16 h, over which time the solid dissolved. A saturated aqueous $NaHSO_3$ solution was added until the purple color disappeared. The reaction mixture was filtered through a pad of Celite which was washed two times with diethyl ether to remove the brown solid. The filtrate was acidified with conc. HCl until pH 1. The reaction was washed with diethyl ether three times, and the combined extracts were dried over anhydrous Na_2SO_4, the solvent was evaporated under reduced pressure, affording compound **15** as a colorless oil without further purification (240.0 mg, 47%).

^{1}H NMR (500 MHz, $CDCl_3$): 3.69 (s, 3H), 2.77 (d, *J* = 16.5 Hz, 1H), 2.58 (d, *J* = 16.4, 1.4 Hz, 1H) 2.38 (t, *J* = 8.1 Hz, 2H), 2.03 (ddd, *J* = 14.1, 9.2, 6.8 Hz, 1H), 1.94 (ddd, *J* = 14.1, 9.4, 6.5 Hz, 1H), 1.30 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$): 179.5, 177.4, 175.9, 52.4, 43.3, 42.5, 33.3, 29.4, 21.7.

To compound **15** (240.0 mg, 1.10 mmol, 1 eq.) in toluene (2.5 mL) was added MeOH (245µL, 6.05 mmol, 5.5 eq.) and cc. H_2SO_4 (91 µL, 1.70 mmol, 1.6 eq.). The reaction was stirred for 16 hours at 90°C and after cooling to room temperature the organic solvent was removed under reduced pressure. The residue was diluted with ethyl acetate and washed with a saturated $NaHCO_3$ solution and brine two times. The organic layer was dried over anhydrous Na_2SO_4, the solvent was evaporated under reduced pressure affording compound **16** as a yellow oil without further purification (163.0 mg, 60%).

LC-MS ESI $[M+H^+]^+$= 247;

^{1}H NMR (500 MHz, $CDCl_3$): 3.70 (s, 3H), 3.67 (s, 3H), 3.66 (s, 3H), 2.76 (d, *J* = 15.9 Hz, 1H), 2.46 (d, *J* = 15.9 Hz, 1H), 2.38 – 2.23 (m, 2H), 2.01 (ddd, *J* = 14.0, 10.3, 6.0 Hz, 1H), 1.89 (ddd, *J* = 14.0, 10.2, 6.4 Hz, 1H), 1.27 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$): 175.9, 173.5, 171.4, 52.2, 51.8, 51.7, 43.7, 42.7, 33.9, 29.5, 21.7.

A solution of KO^{t}Bu (111.4 mg, 0.993 mmol, 1.5 eq.) in anhydrous THF (1 mL) was cooled to 0°C and compound **16** in anhydrous THF (1.5 mL) was added dropwise. The reaction was allowed to stir at 25°C for 1.5 hours, during which time it turned brown. Glacial acetic acid (0.20 mL) was added, resulting in a brownish solution containing a white precipitate. A solution of 0.40 g Na_2HPO_4 in 1.45 mL of water was added, causing the suspension to become homogeneous. The organic layer was separated, and the aqueous layer was washed with chloroform three times. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, the solvent was evaporated under reduced pressure. The resulting crude product was purified by flash chromatography (hexanes: ethyl acetate) to give compound **17** as inseparable diastereomers as a yellow oil (50.0 mg, 35%).

LC-MS ESI $[M+H^+]^+$= 215;

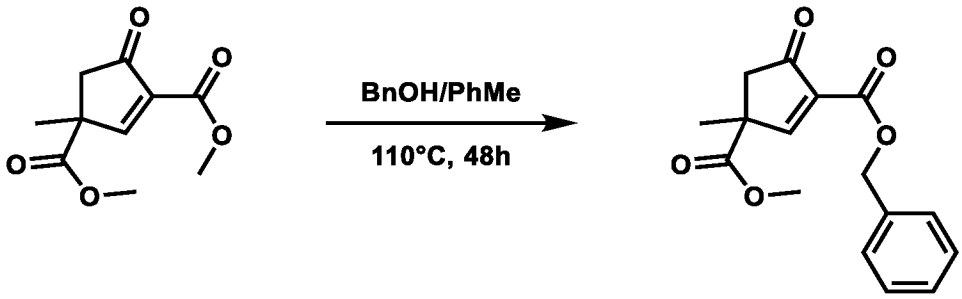
To a solution of compound **17** (50.0 mg, 0.233 mmol, 1 eq.) in methylene chloride (0.1 mL) at 0°C was added pyridine (23µL, 0.280 mmol, 1.2 eq.). After 5 min, a solution of phenylselenyl chloride (53.6 mg, 0.280 mmol, 1.2 eq.) in methylene chloride (0.1 mL) was added dropwise. After 16 hours the reaction mixture was quenched with a saturated aqueous solution of ammonium chloride and washed twice with ethyl acetate. The combined organic layers were washed with water two times and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure affording α–**17**-phenyl Se-adduct intermediate.

To a solution of the above prepared α–**17**-phenyl Se-adduct in methylene chloride (1 mL) was added m-CPBA (138.1 mg, 0.560 mmol, 2.4 eq.) at 0°C in small amounts. After 16 hours, water was added and the mixture was washed three times with methylene chloride. The organic layers were washed with a 5% $NaHCO_3$ solution and brine two times, dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure. The resulting crude product was purified by flash chromatography (hexanes: ethyl acetate) to give compound **18** as a yellow oil (26.3 mg, 53%).

LC-MS ESI $[M+H^+]^+$= 213;

^{1}H NMR (500 MHz, $CDCl_3$): 8.20 (s, 1H), 3.82 (s, 3H), 3.73 (s, 3H), 3.11 (d, *J* = 18.9 Hz, 1H), 2.41 (d, *J* = 18.9 Hz, 1H), 1.53 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$): 200.2, 172.9, 171.9, 161.9, 135.6, 53.2, 52.3, 49.0, 47.2, 24.4.

1-Benzyl 3-methyl 3-methyl-5-oxocyclopent-1-ene-1,3-dicarboxylate: (RU169)

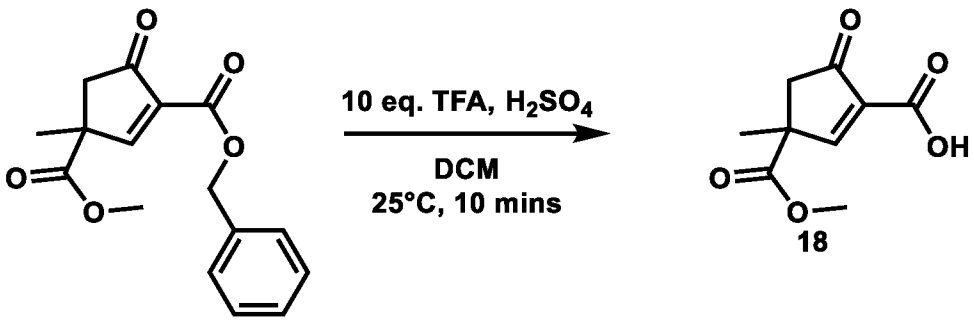


To a solution of compound **18** (120.0 mg, 0.566 mmol, 1 eq.) in toluene (2.4 mL) was added benzyl-alcohol (588 µL, 5.66 mmol, 10 eq.) and the resulting mixture was stirred at 110 °C for 48 hours. After the starting material was fully consumed the volatiles were evaporated under reduced pressure. The crude product was purified by flash chromatography (hexanes: ethyl acetate) to give compound **RU169** as a yellow oil (72.0 mg, 44%).

LC-MS ESI $[M+H^+]^+$= 289;

^{1}H NMR (500 MHz, $CDCl_3$): 8.20 (s, 1H), 7.43 (m, 2H), 7.40 – 7.31 (m, 3H), 5.28 (d, *J* = 2.3 Hz, 2H), 3.74 (s, 3H), 3.14 (d, *J* = 18.9 Hz, 1H), 2.42 (d, = 18.9 Hz, 1H), 1.54 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$): 200.1, 172.8, 171.6, 161.1, 135.4, 135.3, 128.6, 128.43, 128.41, 66.8, 53.1, 48.9, 47.0, 24.3.

3-(Methoxycarbonyl)-3-methyl-5-oxocyclopent-1-ene-1-carboxylic acid: (19)

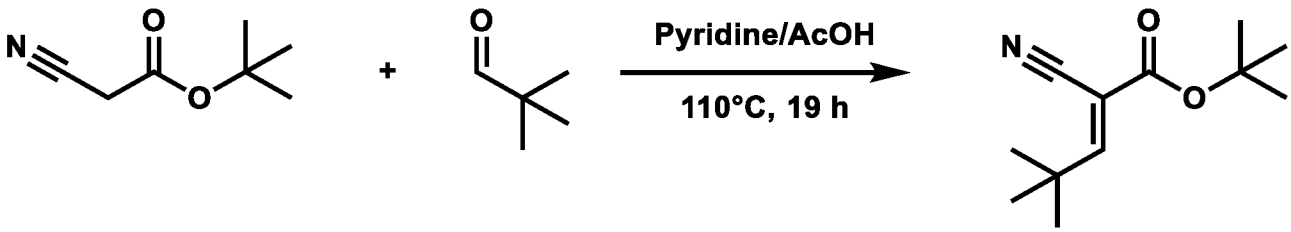


To a solution of compound **RU169** (450.0 mg, 1.56 mmol, 1 eq.) in methylene chloride (9 mL) was added TFA (1.19 mL, 15.6 mmol, 10 eq.) and 98% sulfuric acid (10 drops, 0.5 mL) and the resulting mixture was stirred at 25°C for 10 minutes. After the starting material was consumed, the reaction mixture was diluted with 150 mL of water and was washed with methylene chloride three times. The combined organic layers were washed two times with brine, dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure affording compound **19** as an off-white solid (309.0 mg, 99 %).

LC-MS ESI $[M+H^+]^+$= 199;

^{1}H NMR (500 MHz, $CDCl_3$): 8.48 (s, 1H), 3.78 (s, 3H), 3.32 (d, 1H), 2.57 (d, 1H), 1.61 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$): 206.2, 175.0, 172.1, 161.1, 133.1, 53.3, 49.7, 46.4, 24.0.

***Tert*-butyl (E)-2-cyano-4,4-dimethylpent-2-enoate: (RU150)**

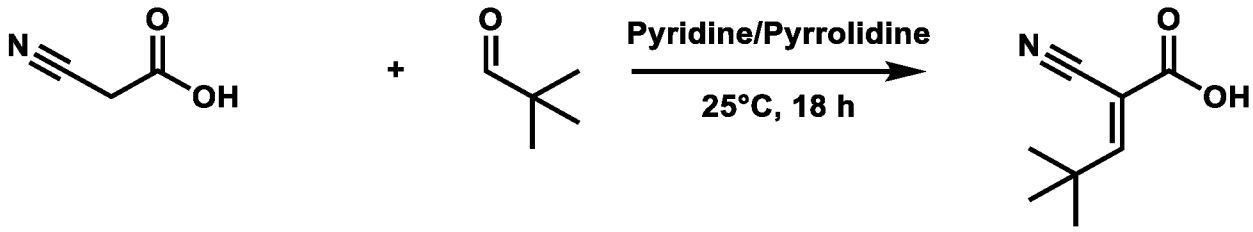


A mixture of pivaldehyde (0.61 g, 7.10 mmol, 1 eq.), tert-butyl 2-cyanoacetate (1.00 g, 7.08 mmol, 1 eq.), acetic acid (406 µL, 7.08 mmol, 1 eq.) and pyridine (573 µL, 7.08 mmol, 1 eq.) was heated at 110°C for 1 hour. A second portion of

pivaldehyde (0.61 g, 7.10 mmol, 1 eq.) was added and the mixture was heated at 110°C for a further 18 hours. After cooling, the mixture was poured into 1M aqueous HCl (10 mL). The resulting mixture was extracted with ethyl acetate (3*10 mL). The combined organic layers were washed with 1M aqueous HCl, dried over anhydrous Na2SO4 and concentrated to give a colorless oil. The oil was purified by flash chromatography to give RU150 as a colorless oil (1.00 g, 68%).

^{1}H NMR (500 MHz, $CDCl_3$): 7.54 (s, 1H), 1.54 (s, 9H), 1.30 (s, 9H).
^{13}C NMR (125 MHz, $CDCl_3$): 170.5, 161.0, 114.4, 107.3, 83.5, 34.8, 28.9, 27.9.

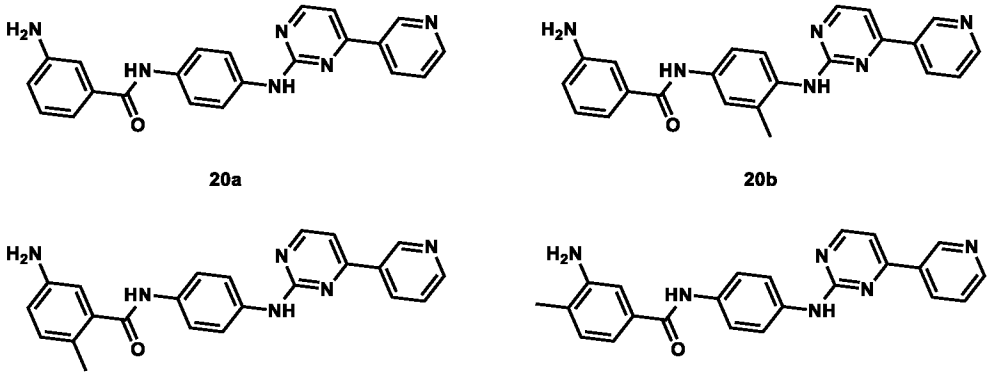
(E)-2-cyano-4,4-dimethylpent-2-enoic acid: (CN-9)



Pivaldehyde (2.03 g, 23.5 mmol, 2 eq.) was added to a solution of 2-cyanoacetic acid (1.0 g, 11.8 mmol) in pyridine (5 mL). Pyrrolidine (201 mg, 2.82 mmol, 0.24 eq.) was added, and the reaction mixture was stirred for 18 hours at 25°C. The mixture was then poured onto conc. HCl (6 mL) and water (10 mL) and extracted with ethyl acetate (3 × 10 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and filtered, and the solvent was evaporated under reduced pressure to give **CN-9** as a white solid (1.53 g, 85%).

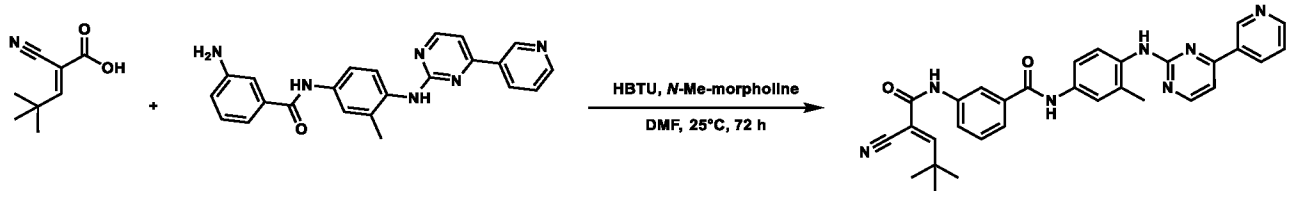
^{1}H NMR (500 MHz, $CDCl_3$): 7.72 (s, 1H), 1.34 (s, 9H).
^{13}C NMR (125 MHz, $CDCl_3$): 174.3, 166.7, 113.6, 105.3, 35.6, 28.9.

IN-7,8,9,10 scaffolds:



The **IN-7, 8, 9, 10 scaffolds (20a-d)** were synthesized according to the literature procedures (see in Qian et al, 2019).

(*E*)-3-(2-cyano-4,4-dimethylpent-2-enamido)-*N*-(3-methyl-4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)benzamide: (RU155-IN-8)

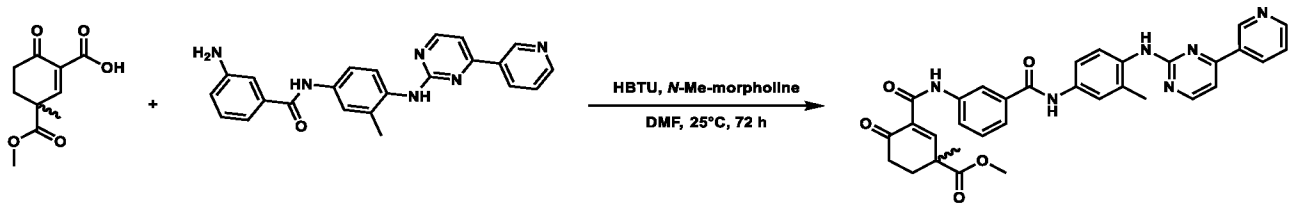


CN-9 (77.3 mg, 0.504 mmol, 2 eq.) was dissolved in anhydrous dimethylformamide (1.0 mL) in a flame-dried vial under a nitrogen atmosphere, HBTU (191.3 mg, 0.504 mmol, 2 eq.) and *N*-methylmorpholine (111 µL, 1.01 mmol, 4.0 eq.) were added and the mixture was stirred at 25°C. After 30 minutes an anhydrous DMF solution (1.0 mL) of compound **20b** (100 mg, 0.252 mmol, 1 eq.) was added and the reaction mixture was stirred at 25°C for 3 days. The reaction was monitored by HPLC-MS and when the starting material was consumed the reaction mixture was diluted with ethyl acetate, washed

two times with a 5% citric acid solution, two times with a saturated aqueous Na_2CO_3 solution, and two times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The resulting crude product was purified by preparative RP-HPLC using a gradient method on a Gemini® 5µm C18 110 Å column (H_2O:MeCN = 95:5 (0.1 % HCOOH) to 100% MeCN (0.1 % HCOOH)). Compound **RU155-IN-8** was obtained as a yellow solid (40.2 mg, 25%).

LC-MS ESI $[M+H^+]^+$= 532;

Methyl 1-methyl-3-((3-((3-methyl-4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)carbamoyl)phenyl)carbamoyl)-4-oxocyclohex-2-ene-1-carboxylate: (BD837-IN-8 and BD838-IN-8)

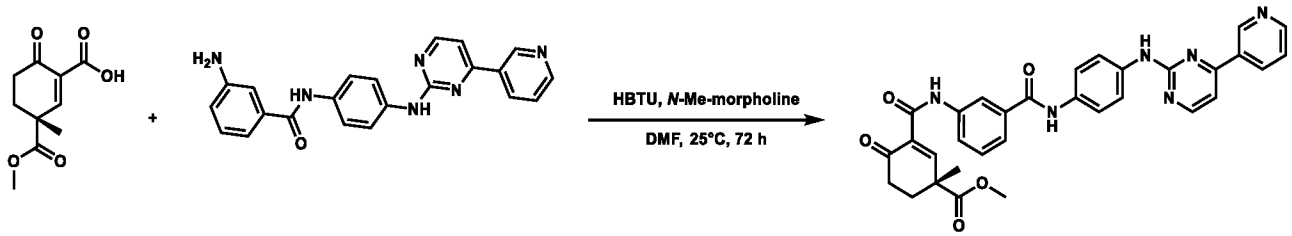


Compound **RU57** (87.5 mg, 0.412 mmol, 1.5 eq.) was dissolved in anhydrous dimethylformamide (1.125 mL) in a flame-dried vial under a nitrogen atmosphere, HBTU (156.0 mg, 0.412 mmol, 1.5 eq.) and *N*-methylmorpholine (91 µL, 0.825 mmol, 3.0 eq.) were added and the mixture was stirred at 25°C. After 30 minutes an anhydrous DMF solution (1.125 mL) of compound **20b** (109 mg, 0.275 mmol, 1 eq.) was added and the reaction mixture was stirred at 25°C for 3 days. The reaction was monitored by HPLC-MS and when the starting material was consumed the reaction mixture was diluted with ethyl acetate, washed two times with a 5% citric acid solution, two times with a saturated aqueous Na_2CO_3 solution, and two times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The resulting crude product was purified by preparative RP-HPLC using a gradient method on a Gemini® 5µm C18 110 Å column (H_2O:MeCN = 95:5 (0.1 % HCOOH) to 100% MeCN (0.1 % HCOOH)). Compound **BD837/838-IN-8** was obtained as a yellow solid (40.2 mg, 25%).

LC-MS ESI $[M+H^+]^+$= 591;

^{1}H NMR (500 MHz, $CDCl_3$): 10.86 (s, 1H), 9.22 (d, *J* = 2.3 Hz, 1H), 8.75 – 8.64 (m, 1H), 8.47 (d, *J* = 5.1 Hz, 1H), 8.37 – 8.32 (m, 1H), 8.26 (s, 1H), 8.21 (t, *J* = 1.9 Hz, 1H), 8.09 (s, 1H), 7.99 (d, *J* = 8.7 Hz, 1H), 7.83 – 7.73 (m, 1H), 7.67 (d, *J* = 7.7 Hz, 1H), 7.62 (d, *J* = 2.5 Hz, 1H), 7.53 – 7.50 (m, 1H), 7.46 – 7.39 (m, 2H), 7.14 (d, *J* = 5.1 Hz, 1H), 6.98 (s, 1H), 3.77 (s, 3H), 2.77 – 2.57 (m, 2H), 2.57 – 2.50 (m, 1H), 2.36 (s, 3H), 2.03 (ddd, *J* = 14.4, 9.9, 5.0 Hz, 1H), 1.55 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$): 199.6, 173.4, 165.3, 162.7, 162.0, 161.1, 160.8, 159.3, 151.6, 148.6, 138.4, 136.1, 134.6, 134.2, 134.0, 132.9, 130.4, 129.6, 129.1, 123.8, 123.7, 123.1, 122.7, 118.8, 118.7, 108.1, 53.1, 45.2, 36.0, 32.1, 24.8, 18.4.

Methyl (*R*)-1-methyl-4-oxo-3-((3-((4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)carbamoyl)phenyl)carbamoyl)cyclohex-2-ene-1-carboxylate: (RU174-IN-7)

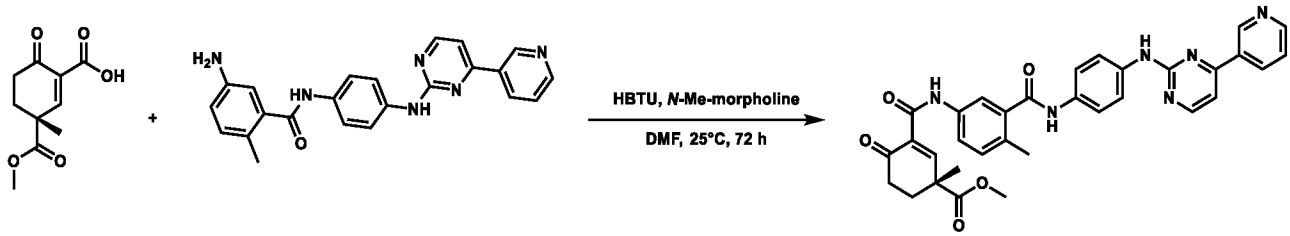


Compound **RU57** (50.0 mg, 0.236 mmol, 1.25 eq.) was dissolved in anhydrous dimethylformamide (0.625 mL) in a flame-dried vial under a nitrogen atmosphere, HBTU (89.4 mg, 0.236 mmol, 1.25 eq.) and *N*-methylmorpholine (83 µL, 0.754 mmol, 4.0 eq.) were added and the mixture was stirred at 25°C. After 30 minutes an anhydrous DMF solution (0.625 mL) of compound **20a** (72.1 mg, 0.189 mmol, 1 eq.) was added and the reaction mixture was stirred at 25°C for 3 days. The reaction was monitored by HPLC-MS and when the starting material was consumed the reaction mixture was diluted with ethyl acetate, washed two times with a 5% citric acid solution, two times with a saturated aqueous Na_2CO_3 solution, and two times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The resulting crude product was purified by preparative RP-HPLC using a gradient method on a Gemini® 5µm C18 110 Å column (H_2O:MeCN = 95:5 (0.1 % HCOOH) to 100% MeCN (0.1 % HCOOH)). Compound **RU174-IN-7** was obtained as a yellow solid (27.1 mg, 25%).

LC-MS ESI $[M+H^+]^+$= 577;

^{1}H NMR (500 MHz, $CDCl_3$): 9.13 (s, 1H), 8.64 – 8.54 (m, 1H), 8.39 (d, J = 5.1 Hz, 1H), 8.34 (d, J = 8.2 Hz, 1H), 8.16 (s, 1H), 8.08 (s, 1H), 7.64 (d, *J* = 7.7 Hz, 2H), 7.62 – 7.60 (m, 2H), 7.54 (d, *J* = 7.6 Hz, 2H), 7.45 – 7.39 (m, 1H), 7.37 (t, *J* = 7.9 Hz, 1H), 7.09 (d, *J* = 5.1 Hz, 1H), 3.70 (s, 3H), 2.60 (dt, *J* = 11.8, 5.8 Hz, 2H), 2.44 (dt, *J* = 12.4, 5.7 Hz, 1H), 1.97 (ddd, J = 14.4, 9.5, 5.5 Hz, 1H), 1.47 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$) partial: 199.4, 173.4, 166.2, 162.2, 162.0, 161.0, 160.2, 158.9, 150.6, 147.8, 137.3, 136.1, 135.9, 135.3, 133.4, 133.2, 129.4, 128.8, 124.4, 124.1, 123.8, 121.4, 120.1, 119.3, 107.9, 52.9, 45.1, 35.7, 31.8, 24.4.

Methyl (*R*)-1-methyl-3-((4-methyl-3-((4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)carbamoyl)phenyl)carbamoyl)-4-oxocyclohex-2-ene-1-carboxylate: (RU175-IN-9)

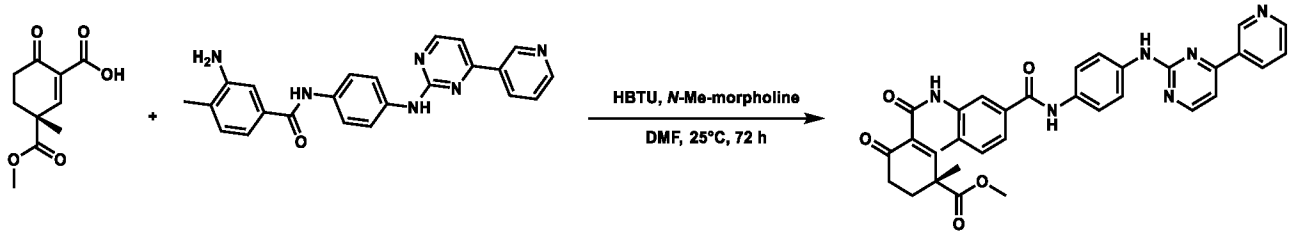


Compound **RU57** (50.0 mg, 0.236 mmol, 1.25 eq.) was dissolved in anhydrous dimethylformamide (0.625 mL) in a flame-dried vial under a nitrogen atmosphere, HBTU (89.4 mg, 0.236 mmol, 1.25 eq.) and *N*-methylmorpholine (83 µL, 0.754 mmol, 4.0 eq.) were added and the mixture was stirred at 25°C. After 30 minutes an anhydrous DMF solution (0.625 mL) of compound **20c** (74.7 mg, 0.189 mmol, 1 eq.) was added and the reaction mixture was stirred at 25°C for 3 days. The reaction was monitored by HPLC-MS and when the starting material was consumed the reaction mixture was diluted with ethyl acetate, washed two times with a 5% citric acid solution, two times with a saturated aqueous Na_2CO_3 solution, and two times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The resulting crude product was purified by preparative RP-HPLC using a gradient method on a Gemini® 5µm C18 110 Å column (H_2O:MeCN = 95:5 (0.1 % HCOOH) to 100% MeCN (0.1 % HCOOH)). Compound **RU175-IN-9** was obtained as a yellow solid (26.4 mg, 24%).

LC-MS ESI $[M+H^+]^+$= 591;

^{1}H NMR (500 MHz, $CDCl_3$): 10.73 (s, 1H), 9.40 (s, 1H), 8.85 (s, 1H), 8.71 (d, *J* = 8.1 Hz, 1H), 8.46 – 8.38 (m, 1H), 8.21 (s, 1H), 8.14 (s, 1H), 7.91 (s, 1H), 7.81 (d, *J* = 8.7 Hz, 1H), 7.68 – 7.59 (m, 4H), 7.44 (dd, *J* = 8.2, 2.2 Hz, 1H), 7.29 (d, *J* = 5.5 Hz, 1H), 7.18 (d, *J* = 8.3 Hz, 1H), 3.76 (s, 3H), 2.75 – 2.59 (m, 2H), 2.51 (dt, *J* = 12.6, 5.8 Hz, 1H), 2.44 (s, 3H), 2.02 (ddd, *J* = 14.3, 9.7, 5.0 Hz, 1H), 1.53 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$) partial: 199.7, 173.4, 167.8, 162.0, 160.8, 139.3, 136.6, 135.7, 134.9, 134.0, 133.1, 132.0, 129.0, 122.5, 121.6, 121.1, 119.2, 107.2, 53.1, 45.2, 35.9, 32.1, 24.7, 19.5.

Methyl (*R*)-1-methyl-3-((2-methyl-5-((4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)carbamoyl)phenyl)carbamoyl)-4-oxocyclohex-2-ene-1-carboxylate: (RU182-IN-10)

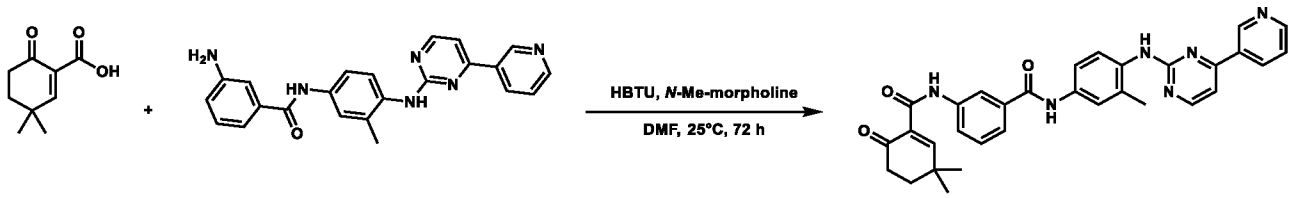


Compound **RU57** (16.1 mg, 0.076 mmol, 1.25 eq.) was dissolved in anhydrous dimethylformamide (0.200 mL) in a flame-dried vial under a nitrogen atmosphere, HBTU (28.7 mg, 0.076 mmol, 1.25 eq.) and *N*-methylmorpholine (27 µL, 0.242 mmol, 4.0 eq.) were added and the mixture was stirred at 25°C. After 30 minutes an anhydrous DMF solution (0.200 mL) of compound **20d** (24.0 mg, 0.061 mmol, 1 eq.) was added and the reaction mixture was stirred at 25°C for 3 days. The reaction was monitored by HPLC-MS and when the starting material was consumed the reaction mixture was diluted with ethyl acetate, washed two times with a 5% citric acid solution, two times with a saturated aqueous Na_2CO_3 solution, and two times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The resulting crude product was purified by preparative RP-HPLC using a gradient method on a Gemini® 5µm C18 110 Å column (H_2O:MeCN = 95:5 (0.1 % HCOOH) to 100% MeCN (0.1 % HCOOH)). Compound **RU182-IN-10** was obtained as a yellow solid (4.4 mg, 12%)

LC-MS ESI $[M+H^+]^+$= 591;

^{1}H NMR (500 MHz, $CDCl_3$): 10.82 (s, 1H), 9.31 (s, 1H), 8.72 (d, J = 1.9 Hz, 1H), 8.50 (d, J = 5.2 Hz, 2H), 8.43 (d, *J* = 8.0 Hz, 2H), 8.30 (s, 1H), 8.07 (s, 1H), 7.58 (s, 1H), 7.51 (s, 1H), 7.72 – 7.61 (m, 2H), 7.33 (d, *J* = 8.0 Hz, 1H), 7.18 (d, *J* = 4.8 Hz, 1H), 3.79 (s, 3H), 2.78 – 2.64 (m, 2H), 2.56 (dt, *J* = 12.5, 5.8 Hz, 1H), 2.44 (s, 3H), 2.06 (ddd, *J* = 14.3, 10.0, 5.0 Hz, 1H), 1.57 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$) partial: 199.9, 173.4, 165.5, 162.8, 162.2, 160.8, 160.2, 158.7, 151.3, 148.3, 136.6, 135.8, 135.1, 133.7, 133.5, 132.2, 131.2, 129.3, 124.5, 124.1, 121.3, 120.3, 119.4, 108.2, 53.2, 45.3, 36.0, 32.2, 24.8, 18.4.

Methyl 1-methyl-3-((3-((3-methyl-4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)carbamoyl)phenyl)carbamoyl)-4-oxocyclohex-2-ene-1-carboxylate: (RU154-IN-8)

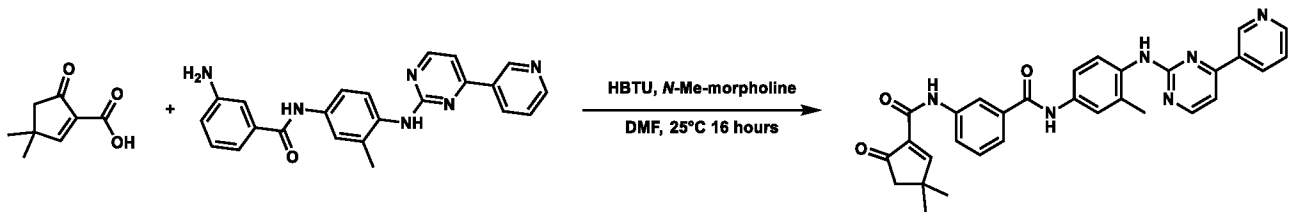


Compound **9** (85.0 mg, 0.504 mmol, 2 eq.) was dissolved in anhydrous dimethylformamide (0.935 mL) in a flame-dried vial under a nitrogen atmosphere, HBTU (192.0 mg, 0.504 mmol, 2 eq.) and *N*-methylmorpholine (111 µL, 1.009 mmol, 4.0 eq.) were added and the mixture was stirred at 25°C. After 30 minutes an anhydrous DMF solution (0.935 mL) of compound **20b** (100 mg, 0.252 mmol, 1 eq.) was added and the reaction mixture was stirred at 25°C for 3 days. The reaction was monitored by HPLC-MS and when the starting material was consumed the reaction mixture was diluted with ethyl acetate, washed two times with a 5% citric acid solution, two times with a saturated aqueous Na_2CO_3 solution, and two times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The resulting crude product was purified by preparative RP-HPLC using a gradient method on a Gemini® 5µm C18 110 Å column (H_2O:MeCN = 95:5 (0.1 % HCOOH) to 100% MeCN (0.1 % HCOOH)). Compound **RU154-IN-8** was obtained as a yellow solid (31.0 mg, 22%).

LC-MS ESI $[M+H^+]^+$= 547;

^{1}H NMR (500 MHz, $CDCl_3$): 10.94 (s, 1H), 9.22 (s, 1H), 8.74 – 8.64 (m, 1H), 8.46 (d, *J* = 5.1 Hz, 1H), 8.34 (dt, *J* = 8.0, 1.9 Hz, 1H), 8.18 (d, *J* = 1.8 Hz, 2H), 8.07 (s, 1H), 7.97 (d, *J* = 8.6 Hz, 1H), 7.78 (ddd, *J* = 8.1, 2.1, 1.1 Hz, 1H), 7.65 (dt, *J* = 7.8, 1.3 Hz, 1H), 7.62 (d, *J* = 2.5 Hz, 1H), 7.52 (dd, *J* = 8.7, 2.5 Hz, 1H), 7.44 – 7.38 (m, 2H), 7.13 (d, *J* = 5.2 Hz, 1H), 7.05 (s, 1H), 2.63 (dd, *J* = 7.5, 6.2 Hz, 2H), 2.34 (s, 3H), 1.92 (t, *J* = 6.8 Hz, 2H), 1.26 (s, 6H).
^{13}C NMR (125 MHz, $CDCl_3$): 200.5, 170.4, 165.4, 162.6, 161.4, 161.0, 159.2, 151.5, 148.5, 138.5, 136.0, 134.7, 134.2, 133.9, 132.8, 130.4, 129.6, 127.5, 123.8, 123.6, 123.5, 123.1, 122.6, 118.74, 118.71, 108.0, 35.7, 35.4, 34.3, 27.3, 18.4.

3-(3,3-dimethyl-5-oxocyclopent-1-ene-1-carboxamido)-*N*-(3-methyl-4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)benzamide: (RU153-IN-8)



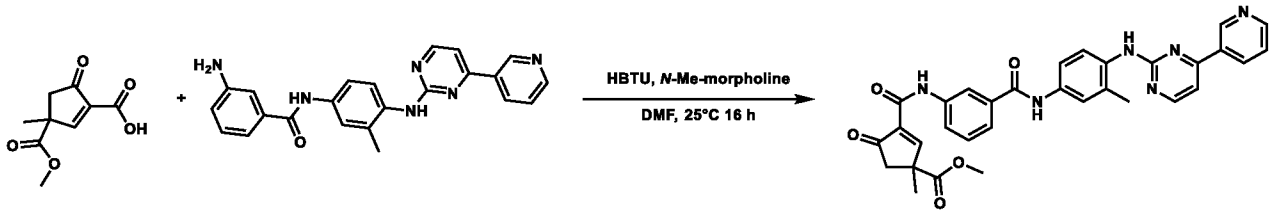
Compound **11** (77.8 mg, 0.504 mmol, 2 eq.) was dissolved in anhydrous dimethylformamide (1.10 mL) in a flame-dried vial under a nitrogen atmosphere, HBTU (191.3 mg, 0.504mmol, 2 eq.) and *N*-methylmorpholine (111 µL, 1.01 mmol, 4 eq.) were added and the mixture was stirred at 25°C. After 30 minutes a DMF solution (1.10 mL) of compound **20b** (100.0 mg, 0.252 mmol, 1 eq.) was added and the reaction mixture was stirred at 25°C for 16 hours. The reaction was monitored by HPLC-MS and when the starting material was consumed the reaction mixture was diluted with ethyl acetate, washed two times with a 5% citric acid solution, two times with a saturated aqueous Na_2CO_3 solution, and two times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The resulting crude product was purified by preparative RP-HPLC using a gradient method on a Gemini® 5µm C18 110 Å column (H_2O:MeCN = 95:5 (0.1 % HCOOH) to 100% MeCN (0.1 % HCOOH)). Compound **RU153-IN-8** was obtained as a yellow solid (39.5 mg, 29%).

LC-MS ESI $[M+H^+]^+$= 533;

^{1}H NMR (500 MHz, $CDCl_3$): 10.06 (s, 1H), 9.22 (s, 1H), 8.73 – 8.65 (m, 1H), 8.46 (d, *J* = 5.1 Hz, 1H), 8.38 (d, *J* = 6.3 Hz, 1H), 8.35 (d, *J* = 8.1 Hz, 1H), 8.18 (d, *J* = 4.5 Hz, 2H), 7.95 (d, *J* = 8.8 Hz, 1H), 7.80 (dd, J = 8.0, 2.0 Hz, 1H), 7.66

(d, J = 7.8 Hz, 1H), 7.62 (d, J = 2.5 Hz, 1H), 7.52 (dd, J = 8.7, 2.5 Hz, 1H), 7.43-7.39 (m, 2H), 7.13 (d, J = 4.5 Hz, 2H), 3.90 (s, 1H), 2.55 (s, 3H), 2.34 (s, 3H), 1.31 (s, 6H).
^{13}C (125 MHz, $CDCl_3$): 207.6, 181.5, 165.3, 162.6, 161.0, 159.2, 159.1, 151.2, 148.3, 138.1, 136.1, 134.9, 134.5, 134.3, 133.9, 130.6, 129.6, 125.8, 124.8, 123.6, 123.4, 123.3, 122.7, 118.8, 118.7, 108.0, 51.5, 39.1, 27.5, 18.4.

Methyl 1-methyl-3-((3-((3-methyl-4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)carbamoyl)phenyl)carbamoyl)-4-oxocyclopent-2-ene-1-carboxylate: (RU172-IN-8)

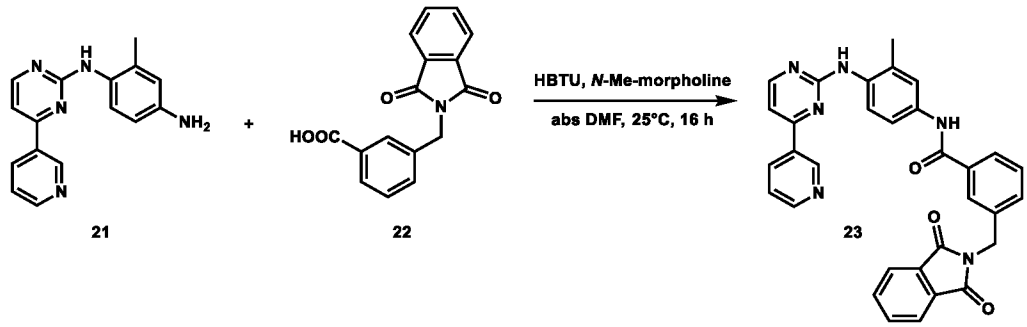


Compound **18** (30.0 mg, 0.151 mmol, 1 eq.) was dissolved in anhydrous dimethylformamide (0.375 mL) in a flame-dried vial under a nitrogen atmosphere, HBTU (57.4 mg, 0.151 mmol, 1 eq.) and *N*-methylmorpholine (75 µL, 0.681 mmol, 4.5 eq.) were added and the mixture was stirred at 25°C. After 30 minutes a DMF solution (0.375 mL) of compound **20b** (90.0 mg, 0.227 mmol, 1.5 eq.) was added and the reaction mixture was stirred at 25°C for 16 hours. The reaction was monitored by HPLC-MS and when the starting material was consumed the reaction mixture was diluted with ethyl acetate, washed two times with a 5% citric acid solution, two times with a saturated aqueous Na_2CO_3 solution and two times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The resulting crude product was purified by preparative RP-HPLC using a gradient method on a Gemini® 5µm C18 110 Å column (H_2O:MeCN = 95:5 (0.1 % HCOOH) to 100% MeCN (0.1 % HCOOH)). Compound **RU172-IN-8** was obtained as a yellow solid (22.2 mg, 25%).

LC-MS ESI $[M+H^+]^+$= 577;

^{1}H NMR (500 MHz, $CDCl_3$): 9.31 (s, 1H), 8.80 (s, 1H), 8.55 – 8.5 (m, 1H), 8.50 (s, 1H), 8.45 – 8.42 (m, 1H), 8.24-8.22 (m, 1H), 7.76 (d, J = 8.7 Hz, 1H), 7.73 (d, J = 1.8 Hz, 1H), 7.72 – 7.71 (m, 1H), 7.67-7.65 (m, 1H), 7.61 (d, J = 2.4 Hz, 1H), 7.59 (d, J = 2.3 Hz, 1H), 7.49 (t, J = 7.9 Hz, 1H), 7.24 (d, J = 5.6 Hz, 1H), 3.78 (s, 3H), 3.34 (d, J = 19.3 Hz, 1H), 2.59 (d, J = 19.3 Hz, 1H), 2.37 (s, 3H), 1.62 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$): 205.5, 173.3, 172.5, 165.7, 158.6, 158.5, 137.0, 136.9, 136.1, 136.0, 135.8, 129.6, 125.0, 123.7, 123.6, 122.8, 122.6, 119.1, 119.0, 118.7, 118.6, 107.1, 53.2, 49.8, 49.6, 49.4, 49.3, 49.1, 49.0, 47.3, 24.1, 18.2.

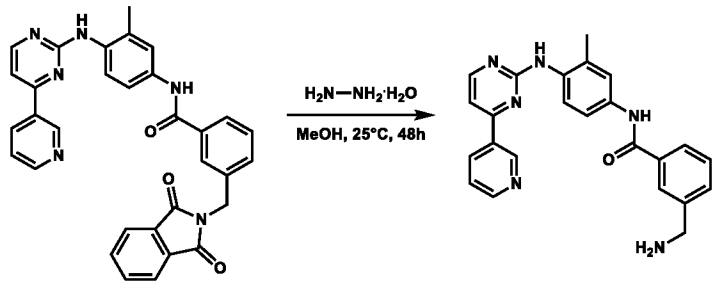
3-((1,3-dioxoisoindolin-2-yl)methyl)-*N*-(3-methyl-4-((4-pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)benzamide: (23)



Compound **22** (0.140 g, 0.50 mmol) was dissolved in anhydrous DMF (3 mL) under a nitrogen atmosphere and HBTU (0.192 g, 0.50 mmol) and *N*-methyl-morpholine (504 µl, 0.455 g, 1.5 mmol) were added to the solution. Then the reaction mixture was stirred for 10 minutes and compound **21** (0.110 g, 0.40 mol) was added. After that, the reaction mixture was stirred overnight at room temperature. After that, the reaction mixture was diluted with ethyl acetate and was washed with a 10% citric acid solution two times and brine four times. The organic phase was dried over Na_2SO_4. Then it was filtered and evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel using methylene chloride: methanol = 9:1 as eluent. Compound **23** was isolated as yellow crystals (0.12 g, 44%).
m.p. 118-123 °C.

^{1}H NMR (500 MHz, $CDCl_3$): δ 9.26 (s, 1H), 8.72 (s, 1H), 8.49 (s, 1H), 8.39 (s, 1H), 8.15 (s, 1H), 8.02-7.17 (m, 13H), 4.92 (s, 2H), 2.38 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$): 169.2, 167.9, 162.3, 160.3, 158.1, 149.9, 147.1, 136.9, 136.6, 136.0, 135.5, 134.0, 133.0, 132.0, 131.3, 130.8, 129.8, 129.4, 129.1, 128.7, 126.5, 125.0, 123.8, 123.4, 122.6, 118.6, 118.3, 110.3, 107.5, 41.2, 38.5.

3-(aminomethyl)-*N*-(3-methyl-4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)benzamide: (24)

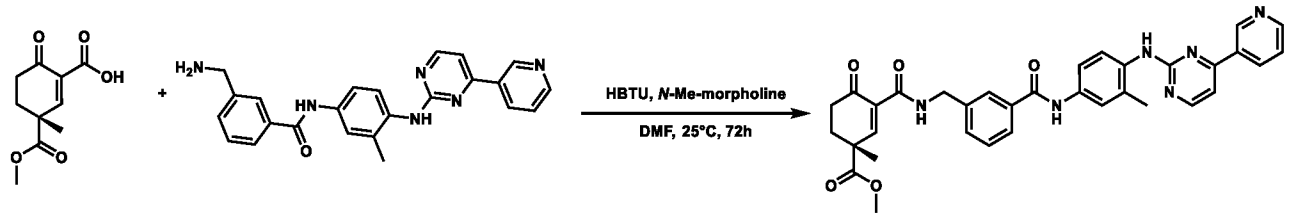


24

Compound **23** (0.120 g, 0.2 mol) was dissolved in methanol (2 mL) and hydrazine hydrate (22 µl, 0.022 g, 0.7 mol) was added to the solution. After that the reaction mixture was stirred at room temperature for 48 hours. The white suspension formed was filtered and the filtrate was evaporated under reduced pressure. The crude product **24** was isolated as yellow crystals (0.02 g, 22%) and used for next step without further purification.

^{1}H NMR (500 MHz, CD_3OD): δ 9.24 (br, 1H), 8.64 (br, 1H), 8.49 (d, *J* = 7.0 Hz, 1H), 8.43 (d, *J* = 5.2 Hz, 1H), 8.20-8.18 (m, 1H), 8.04 (s, 1H), 7.99 (d, *J* = 8.7 Hz, 1H), 7.89-7.88 (m, 1H), 7.66 (d, *J* = 7.0 Hz, 1H), 7.60 (s, 1H), 7.59 (t, *J* = 8.7 Hz, 2H), 7.56-7.54 (m, 1H), 7.32 (d, *J* = 7.0 Hz, 1H), 7.26 (t, *J* = 5.8 Hz, 1H), 4.22 (br, 2H), 3.35 (s, 2H), 2.34 (s, 3H).
^{13}C NMR (125 MHz, CD_3OD): δ 167.9, 163.6, 162.9, 160.3, 151.8, 148.9, 137.3, 136.6, 135.5, 134.4, 133.9, 133.1, 130.4, 129.4, 128.9, 126.5, 125.1, 124.2, 120.2, 118.6, 112.2, 108.5, 44.1, 18.5.

Methyl (*R*)-1-methyl-3-((3-((3-methyl-4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)carbamoyl)benzyl)carbamoyl)-4-oxocyclohex-2-ene-1-carboxylate: (RU135-IN-8)

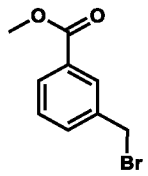


Compound **RU57** (23.3mg, 0.110 mmol, 1.5 eq.) was dissolved in anhydrous dimethylformamide (0.25 mL) in a flame-dried vial under a nitrogen atmosphere, HBTU (41.6 mg, 0.110mmol, 1.5 eq.) and *N*-methylmorpholine (24µL, 0.220 mmol, 3 eq.) were added and the mixture was stirred at 25°C. After 30 minutes a DMF solution (0.25 mL) of compound **24** (30.0 mg, 0.073mmol, 1 eq.) was added and the reaction mixture was stirred at 25°C for 3 days. The reaction was monitored by HPLC-MS and when the starting material was consumed the reaction mixture was diluted with ethyl acetate, washed two times with a 5% citric acid solution, two times with a saturated aqueous Na_2CO_3 solution, and two times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The resulting crude product was purified by preparative RP-HPLC using a gradient method on a Gemini® 5µm C18 110 Å column (H_2O:MeCN = 95:5 (0.1 % HCOOH) to 100% MeCN (0.1 % HCOOH)). Compound **RU135-IN-8** was obtained as a yellow solid (13.3 mg, 30%).

LC-MS ESI $[M+H^+]^+$= 605;

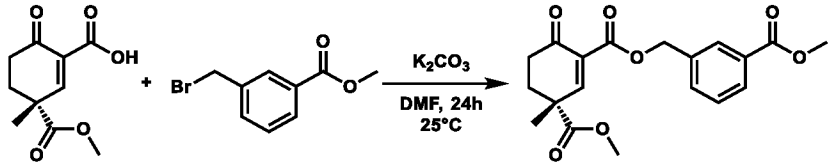
^{1}H NMR (500 MHz, $CDCl_3$): 9.09 (t, *J* = 6.3 Hz, 1H), 8.47 (d, *J* = 8.0 Hz, 2H), 8.17 (s, 1H), 7.98 – 7.88 (m, 2H), 7.84 (s, 1H), 7.79 (d, *J* = 7.8 Hz, 1H), 7.64 (d, *J* = 2.3 Hz, 1H), 7.56 – 7.48 (m, 1H), 7.46 (t, *J* = 7.6 Hz, 1H), 7.19 (d, *J* = 5.0 Hz, 1H), 4.64 (t, *J* = 6.1 Hz, 2H), 3.76 (s, 3H), 2.69 – 2.57 (m, 1H), 2.54 – 2.47 (m, 1H), 2.38 (s, 3H), 2.04 – 1.95 (m, 1H), 1.52 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$) partial: 199.1, 173.4, 165.4, 164.3, 162.8, 160.8, 160.0, 157.6, 139.1, 135.7, 135.4, 134.5, 133.1, 131.1, 131.0, 129.1, 128.9, 126.2, 126.0, 123.5, 122.4, 118.4, 107.6, 52.8, 44.9, 43.2, 35.8, 32.0, 24.6, 18.3.

2-methyl-*N*-(4-(pyridin-3-yl)pyrimidin-2-yl)benzene-1,4-diamine: (21)



(see in Schierle et al., 2020)

1-(3-methoxycarbonyl)benzyl 3-methyl (*R*)-3-methyl-6-oxocyclohex-1-ene-1,3-dicarboxylate: (27)



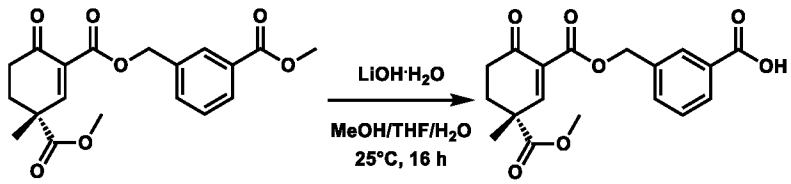
27

Compound **RU57** (0.100 g, 0.50 mmol) was dissolved in anhydrous DMF (2 mL) under a nitrogen atmosphere and K_2CO_3 (0.064 g, 0.47 mmol) was added to the solution and the mixture was stirred for 45 minutes. Then 3-methoxycarbonylbenzyl bromide (0.09 g, 0.39 mmol) was added to the reaction mixture and was stirred for 24 hours at room temperature. After that, the reaction mixture was diluted with ethyl acetate and was washed with brine four times. The combined organic phases were dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel using hexane: ethyl acetate = 3:1 as eluent. The product **27** was isolated as a yellow oil (0.04 g, 23%).

LC-MS ESI $[M+H^+]^+$= 361;

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.03 (d, *J* = 11.7 Hz, 2H), 7.58 (d, *J* = 6.8 Hz, 1H), 7.46 (t, *J* = 7.5 Hz, 1H), 5.30 (s, 2H), 3.93 (s, 3H), 3.79 (s, 3H), 2.60-2.55 (m, 2H), 2.37-2.23 (m, 2H), 1.43 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): 198.1, 174.1, 166.2, 164.5, 156.2, 135.2, 134.7, 132.6, 130.6, 129.7, 129.2, 128.8, 67.0, 64.5, 52.7, 52.2, 42.0, 33.0, 25.8.

(*R*)-3-(((3-methoxycarbonal)-3-methyl-6-oxocyclohex-1-ene-1-carbonyl)oxy)methyl)benzoic acid: (28)



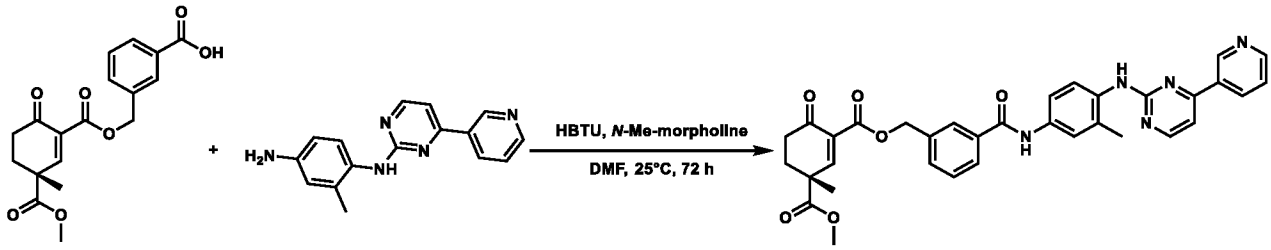
28

Compound **27** (0.04 g, 0.10 mol) was dissolved in THF:MeOH:H_2O (3:1:1) (0.6 : 0.2 : 0.2 mL) and $LiOH{\cdot}H_2O$ (0.014 g, 0.33 mmol) was added. Then the reaction mixture was stirred overnight at room temperature. After that, the reaction mixture was diluted with distilled water and was washed with methylene chloride two times. The aqueous phase was acidified with a 10% citric acid solution and was washed with methylene chloride three times. The combined organic phases were dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure. The product **28** was isolated as a yellow oil (0.015 g, 43%).

LC-MS ESI $[M+H^+]^+$= 347;

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.14 (s, 1H), 8.07 (d, *J* = 7.9 Hz, 1H), 7.70 (d, *J* = 7.9 Hz, 1H), 7.56 (s, 1H), 7.50 (t, *J* = 7.7 Hz, 1H), 5.32 (s, 2H), 3.77 (s, 3H), 2.52-2.46 (m, 2H), 2.03-1.98 (m, 2H), 1.26 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$): δ 193.2, 173.5, 164.0, 136.1, 133.4, 132.2, 131.4, 130.0, 129.7, 129.5, 128.9, 128.5, 66.3, 52.9, 44.9, 35.3, 32.7, 24.5.

3-methyl 1-(3-((3-methyl-4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)carbamoyl)benzyl) (*R*)-3-methyl-6-oxocyclohex-1-ene-1,3-dicarboxylate: (RU137-IN-8)

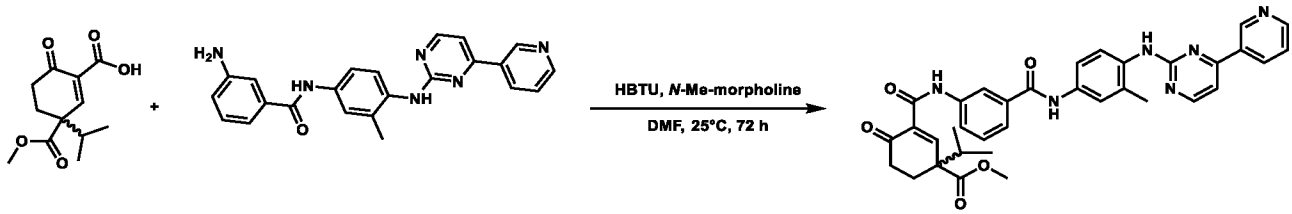


Compound **28** (0.02 g, 0.06 mmol) was dissolved in anhydrous DMF (1 mL) under a nitrogen atmosphere and HBTU (0.022 g, 0.06 mmol) and *N*-methyl-morpholine (0.052 g, 1.74 mmol) were added to the solution. Then the reaction mixture was stirred for 10 minutes and compound **21** (0.016 g, 0.06 mmol) was added. After that, the reaction mixture was stirred overnight at room temperature. Then the reaction mixture was diluted with ethyl acetate and washed two times with a 5% citric acid solution, two times with a saturated aqueous Na_2CO_3 solution, and two times with brine. The organic phase was dried over Na_2SO_4. Then it was filtered and evaporated under reduced pressure. The resulting crude product was purified by preparative RP-HPLC using a gradient method on a Gemini® 5µm C18 110 Å column (H_2O:MeCN = 95:5 (0.1 % HCOOH) to 100% MeCN (0.1 % HCOOH)). Compound **RU137-IN-8** was obtained as a yellow oil (0.003 g, 8%).

LC-MS ESI $[M+H^+]^+$= 606;

^{1}H NMR (500 MHz, $CDCl_3$): 8.47 (s, 1H), 8.42 (d, *J* = 7.8 Hz, 1H), 8.38 (s, 1H), 8.14 (s, 1H), 7.94 (d, *J* = 7.5 Hz, 1H), 7.90 (d, *J* = 8.6 Hz, 1H), 7.74 (s, 1H), 7.64 (d, *J* = 8.9Hz, 1H), 7.56 (s, 1H), 7.54 – 7.48 (m, 2H), 7.18 (d, *J* = 5.3 Hz, 1H), 5.39 (s, 2H), 3.77 (s, 3H), 2.69 – 2.56 (m, 2H), 2.54-2.49 (m, 1H), 2.38 (s, 3H), 2.10 – 1.94 (m, 1H), 1.52 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$) partial: 194.1, 173.5, 172.8, 165.2, 164.6, 163.1, 160.4, 157.9, 156.5, 150.8, 147.9, 136.5, 135.4, 135.0, 133.3, 132.2, 131.2, 130.5, 129.2, 127.5, 125.7, 123.8, 122.4, 118.5, 107.7, 66.3, 53.1, 44.5, 35.5, 32.1, 24.7, 18.5.

Methyl 1-isopropyl-3-((3-((3-methyl-4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)carbamoyl)phenyl)carbamoyl)-4-oxocyclohex-2-ene-1-carboxylate: (RU210-IN-8 and RU211-IN-8)

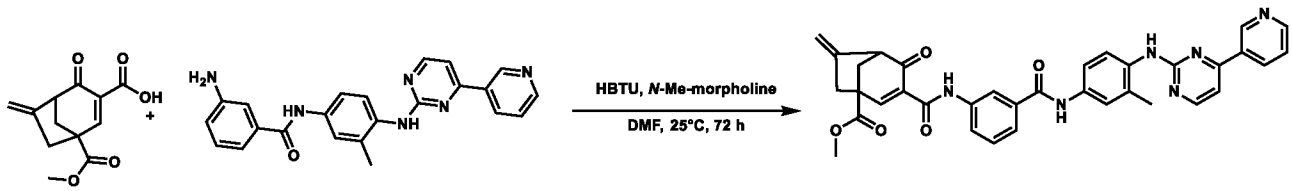


Compound **9** (13.9 mg, 0.058 mmol, 1.25 eq.) was dissolved in anhydrous dimethylformamide (0.154 mL) in a flame-dried vial under a nitrogen atmosphere, HBTU (21.9 mg, 0.058 mmol, 1.25 eq.) and *N*-methylmorpholine (13 µL, 0.116 mmol, 2.5 eq.) were added and the mixture was stirred at 25°C. After 30 minutes an anhydrous DMF solution (0.154 mL) of compound **20b** (20.0 mg, 0.046 mmol, 1 eq.) was added and the reaction mixture was stirred at 25°C for 3 days. The reaction was monitored by HPLC-MS and when the starting material was consumed the reaction mixture was diluted with ethyl acetate, washed two times with a 5% citric acid solution, two times with a saturated aqueous Na_2CO_3 solution, and two times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The resulting crude product was purified by preparative RP-HPLC using a gradient method on a Gemini® 5µm C18 110 Å column (H_2O:MeCN = 95:5 (0.1 % HCOOH) to 100% MeCN (0.1 % HCOOH)). Compound **RU210/211-IN-8** was obtained as a yellow solid (15.6 mg, 51%).

LC-MS ESI $[M+H^+]^+$= 619;

^{1}H NMR (500 MHz, $CDCl_3$): 10.91 (s, 1H), 9.26 (s, 1H), 8.73 (s, 1H), 8.48 (d, J = 5.1 Hz, 1H), 8.35 (d, J = 7.9 Hz, 1H), 8.27 (d, J = 1.8 Hz, 1H), 8.25 (s, 1H), 8.02 – 7.96 (m, 2H), 7.79 (dt, J = 8.0, 1.5 Hz, 1H), 7.69 – 7.66 (m, 1H), 7.62 (d, J = 2.5 Hz, 1H), 7.52 (dd, J = 8.7, 2.6 Hz, 1H), 7.45 (t, J = 7.9 Hz, 1H), 7.15 (d, J = 5.1 Hz, 1H), 6.98 (s, 1H), 5.29 (s, 1H), 3.76 (s, 3H), 2.76 – 2.57 (m, 2H), 2.51 – 2.41 (m, 1H), 2.37 (s, 3H), 2.04 (td, J = 13.3, 5.4 Hz, 1H), 0.98 (dd, J = 14.0, 6.9 Hz, 6H).

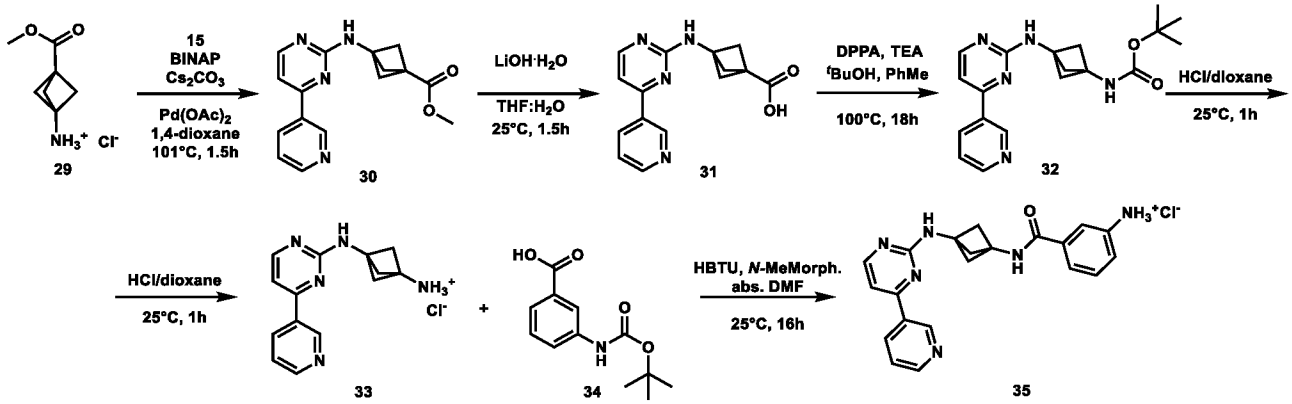
Methyl 3-((3-((3-methyl-4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)carbamoyl)phenyl)carbamoyl)-6-methylene-4-oxobicyclo[3.2.1]oct-2-ene-1-carboxylate (RU212-IN-8 and RU213-IN-8)



Compound **10** (15.0 mg, 0.064 mmol, 1.25 eq.) was dissolved in anhydrous dimethylformamide (0.170 mL) in a flame-dried vial under a nitrogen atmosphere, HBTU (24.1 mg, 0.064 mmol, 1.25 eq.) and *N*-methylmorpholine (14 µL, 0.127 mmol, 2.5 eq.) were added and the mixture was stirred at 25°C. After 30 minutes an anhydrous DMF solution (0.170 mL) of compound **20b** (22 mg, 0.051 mmol, 1 eq.) was added and the reaction mixture was stirred at 25°C for 3 days. The reaction was monitored by HPLC-MS and when the starting material was consumed the reaction mixture was diluted with ethyl acetate, washed two times with a 5% citric acid solution, two times with a saturated aqueous Na_2CO_3 solution, and two times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The resulting crude product was purified by preparative RP-HPLC using a gradient method on a Gemini® 5µm C18 110 Å column (H_2O:MeCN = 95:5 (0.1 % HCOOH) to 100% MeCN (0.1 % HCOOH)). Compound **RU212/213-IN-8** was obtained as a yellow solid (9.7 mg, 31%).

LC-MS ESI $[M+H^+]^+$= 615;

3-((3-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)bicyclo[1.1.1]pentan-1-yl)carbamoyl)benzenaminium chloride: (35)



Compound **29** was synthesized according to the literature procedures (Kokhan et al., 2017).

Compound **29** (541.0 mg, 2.823 mmol) was dissolved in anhydrous 1,4-dioxane (18 mL) and $PdOAc_2$ (58.87 mg, 0.282 mmol), BINAP (351.6 mg, 0.565 mmol), Cs_2CO_3 (2.073 g, 8.469 mmol) and compound **33** (0.752 g, 4.235 mmol) were added to the solution. After that the reaction mixture was refluxed for 1.5 hours. The reaction mixture was cooled down to room temperature and filtered through Celite and the filtrate was evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel using hexanes:ethyl acetate as eluent. Compound **30** was isolated as an off-white solid (0.460 g, 55%).

LC-MS ESI $[M+H^+]^+$= 297;

^{1}H NMR (500 MHz, $CDCl_3$): 9.25 – 9.24 (d, *J* = 1.8 Hz, 1H), 8.71 (dd, *J* = 4.8, 1.7 Hz, 1H), 8.38 (d, *J* = 5.2 Hz, 1H), 8.31 (dt, *J* = 8.0, 2.0 Hz, 1H), 7.42 (ddd, *J* = 7.9, 4.8, 0.9 Hz, 1H), 7.07 (d, J = 5.2 Hz, 1H), 5.68 (s, 1H), 3.72 (s, 3H), 2.50 (s, 6H).
^{13}C NMR (125 MHz, $CDCl_3$): 170.5, 162.5, 162.5, 159.0, 151.5, 148.7, 134.5, 133.1, 123.7, 107.4, 54.4, 51.9, 47.0, 36.3

Compound **30** (273.0 mg, 0.921 mmol) was dissolved in a mixture of THF and H_2O (7:3, 1.70:0.730 mL), and $LiOH·H_2O$ (57.98 mg, 1.382 mmol, 1.5 eq.) was added to the solution. The mixture was stirred for 1.5 h at room temperature and then diluted with water (10 mL) and washed with methylene chloride (5 mL) two times. The aqueous phase was acidified to pH=7 with a 10% HCl solution and the resulting white precipitate was filtered and dried under reduced pressure affording compound **31** (0.208 g, 80%).

LC-MS ESI $[M+H^+]^+$= 283;

^{1}H NMR (500 MHz, DMSO-d6): 12.37 (s, 1H), 9.28 (d, *J* = 2.2 Hz, 1H), 8.70 (dd, *J* = 4.8, 1.6 Hz, 1H), 8.46 – 8.37 (m, 2H), 8.05 (s, 1H), 7.57 (dd, *J* = 8.0, 4.8 Hz, 1H), 7.32 (d, *J* = 5.2 Hz, 1H), 2.33 (s, 6H).
^{13}C NMR (125 MHz, DMSO-d6): 171.0, 162.3, 161.2, 159.3, 151.2, 147.9, 134.2, 132.5, 123.9, 106.6, 53.4, 46.3, 36.2.

To compound **31** (150.0 mg, 0.531 mmol, 1 eq), in anhydrous toluene (2.5 mL), was added TEA (222 µL, 1.594 mmol, 3.0 eq.) and tert-butanol 176 µL, 1.860 mmol, 3.5 eq.) to this mixture was added DPPA (219.3 mg, 0.797 mmol, 1.5 eq.). The reaction mixture was stirred at 100°C for 16h. After the reaction was completed, the mixture was cooled to room temperature and it was concentrated under reduced pressure to remove the solvent. Water was added to the system, the brownish-white precipitate was filtered, washed with water, and dried under reduced pressure to afford the Boc-protected amine compound **32** as a yellow powder (39.3 mg, 21%).

LC-MS ESI $[M+H^+]^+$= 354;

^{1}H NMR (500 MHz, $CDCl_3$): 9.11 (s, 1H), 8.56 (d, *J* = 4.6 Hz, 1H), 8.29 – 8.19 (m, 2H), 7.38 (dd, *J* = 8.0, 4.9 Hz, 1H), 6.98 (d, *J* = 5.2 Hz, 1H), 3.64 (s, 6H), 2.40 (s, 9H).
^{13}C NMR (125 MHz, $CDCl_3$) partial: 162.2, 162.1, 158.6, 156.3, 153.2, 150.6, 147.9, 135.0, 133.3, 124.0, 106.9, 54.5, 45.5, 45.2.

To the Boc-protected amine compound (50.0 mg, 141.5 mmol) was added 8M solution of HCl in 1,4-dioxane (885 µL, 7.073 mmol, 50 eq.) and the mixture was stirred at 25°C for 1 h. After the starting material was consumed the reaction mixture was evaporated under reduced pressure, suspended in diethyl ether, filtered, and washed two times with diethyl ether affording compound **33** as a yellow solid (38.4 mg, 94%).

LC-MS ESI $[M+H^+]^+$= 254;

^{1}H NMR (500 MHz, DMSO-d6+$CDCl_3$): 9.47 (d, *J* = 2.0 Hz, 1H), 9.10 – 9.02 (m, 2H), 9.00 (d, *J* = 5.5 Hz, 1H), 8.52 (dd, *J* = 5.3, 1.7 Hz, 1H), 8.11 (t, *J* = 3.6, 3.0 Hz, 1H), 7.48 (d, *J* = 5.3 Hz, 1H), 2.39 (s, 3H), 2.36 (s, 3H).
^{13}C NMR (125 MHz, DMSO-d6+$CDCl_3$): 153.3, 140.2, 133.9, 127.7, 125.3, 118.7, 118.6, 105.7, 105.3, 52.6, 51.8, 43.4.

Compound **34** was synthesized according to the literature procedure (Fanrong et al, 2001).

Compound **34** (47.3 mg, 0.199 mmol, 1.5 eq.) was dissolved in anhydrous dimethylformamide (0.500 mL) in a flame-dried vial under a nitrogen atmosphere, HBTU (75.6 mg, 0.199 mmol, 1.5 eq.) and *N*-methylmorpholine (51 µL, 0.465 mmol, 3.5 eq.) were added and the mixture was stirred at 25°C. After 30 minutes compound **37** (38.4 mg, 0.133 mmol, 1 eq.) was added and the reaction mixture was stirred at 25°C for 16 h. The reaction was monitored by HPLC-MS and when the starting material was consumed the reaction mixture was diluted with ethyl acetate, washed two times with a 5% citric acid solution, two times with a saturated aqueous Na_2CO_3 solution, and two times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The resulting crude product was purified by flash column chromatography on silica gel using hexanes:ethyl acetate as eluent, affording the Boc-protected amine as an off-white solid (24.9 mg, 40%).

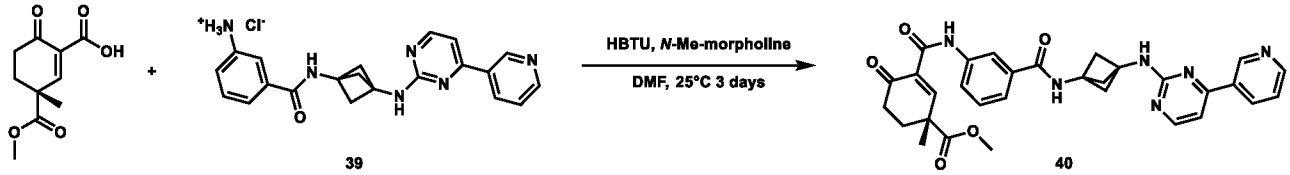
LC-MS ESI $[M+H^+]^+$= 473;

^{1}H NMR (500 MHz, $CDCl_3$): 9.08 (s, 1H), 8.53 (d, *J* = 3.8 Hz, 1H), 8.31 (d, *J* = 8.3 Hz, 1H), 8.24 (d, *J* = 5.2 Hz, 1H), 7.63 (s, 1H), 7.40 – 7.35 (m, 3H), 7.22 (t, *J* = 7.9 Hz, 1H), 6.97 (d, *J* = 5.1 Hz, 1H), 2.49 (s, 6H), 1.41 (s, 9H).
^{13}C NMR (125 MHz, $CDCl_3$) partial: 168.6, 162.2, 162.1, 158.6, 153.7, 150.6, 147.8, 138.8, 135.1, 134.9, 133.4, 129.1, 124.0, 121.8, 117.0, 106.8, 80.6, 54.7, 46.0, 45.5, 28.2.

To the Boc-protected amine compound (24.9 mg, 0.053 mmol) was added an 8M solution of HCl in 1,4-dioxane (329 µL, 2.635 mmol, 50 eq.) and the mixture was stirred at 25°C for 1 h. After the starting material was consumed the reaction mixture was evaporated under reduced pressure, suspended in diethyl ether, filtered, and washed two times with diethyl ether affording compound **35** as a yellow solid (16.9 mg, 78%).

LC-MS ESI $[M+H^+]^+$= 410;

Methyl ®-1-methyl-4-oxo-3-((3-((3-((4-(63yridine-3-yl)pyrimidin-2-yl)amino)bicyclo[1.1.1]pentan-1-yl)carbamoyl)phenyl)carbamoyl)cyclohex-2-ene-1-carboxylate: (RU159-IN-7_isoPHEN)



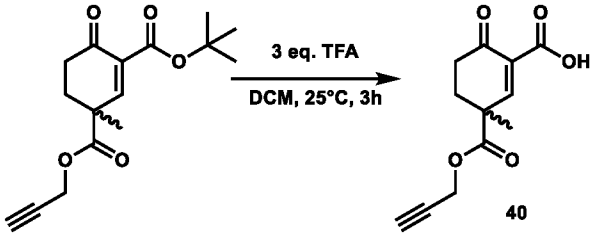
39 40

Compound **RU57** (13.2 mg, 0.062 mmol, 1.5 eq.) was dissolved in anhydrous dimethylformamide (0.100 mL) in a flame-dried vial under a nitrogen atmosphere, HBTU (23.5 mg, 0.062 mmol, 1.5 eq.) and *N*-methylmorpholine (18 µL, 0.165 mmol, 4 eq.) were added and the mixture was stirred at 25°C. After 30 minutes an anhydrous DMF solution (0.150 mL) of compound **39** (16.9 mg, 0.041 mmol, 1 eq.) was added and the reaction mixture was stirred at 25°C for 3 days. The reaction was monitored by HPLC-MS and when the starting material was consumed the reaction mixture was diluted with ethyl acetate, washed two times with a 5% citric acid solution, two times with a saturated aqueous Na_2CO_3 solution, and two times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The resulting crude product was purified by preparative RP-HPLC using a gradient method on a Gemini® 5µm C18 110 Å column (H_2O:MeCN = 95:5 (0.1 % HCOOH) to 100% MeCN (0.1 % HCOOH)). Compound **RU159-IN-7_isoPHEN** was obtained as a yellow oil (12.9 mg, 55%).

LC-MS ESI $[M+H^+]^+$= 567;

^{1}H NMR (500 MHz, $CDCl_3$+CD_3OD): 9.14 (s, 1H), 8.60 (s, 1H), 8.36 (d, *J* = 8.2 Hz, 1H), 8.30 (d, *J* = 5.2 Hz, 1H), 8.19 (s, 1H), 8.04 (t, *J* = 1.9 Hz, 1H), 7.59 (d, *J* = 7.8, 1.4 Hz, 1H), 7.55 – 7.51 (m, 1H), 7.46 – 7.40 (m, 1H), 7.37 (t, *J* = 7.9 Hz, 1H), 7.02 (d, *J* = 5.1 Hz, 1H), 3.73 (s, 3H), 2.67 – 2.59 (m, 2H), 2.56 (s, 6H), 2.51-244 (m, 1H), 2.28 – 2.19 (m, 1H), 1.51 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$+CD_3OD) partial: 199.4, 174.1, 173.4, 168.2, 162.3, 162.2, 161.9, 160.9, 158.7, 150.6, 147.9, 137.3, 135.3, 135.1, 129.3, 128.8, 124.1, 123.6, 119.0, 106.8, 54.7, 52.9, 46.0, 45.1, 42.6, 35.7, 31.8, 24.4.

3-Methyl-6-oxo-3-((prop-2-yn-1-yloxy)carbonyl)cyclohex-1-ene-1-carboxylic acid: (40)



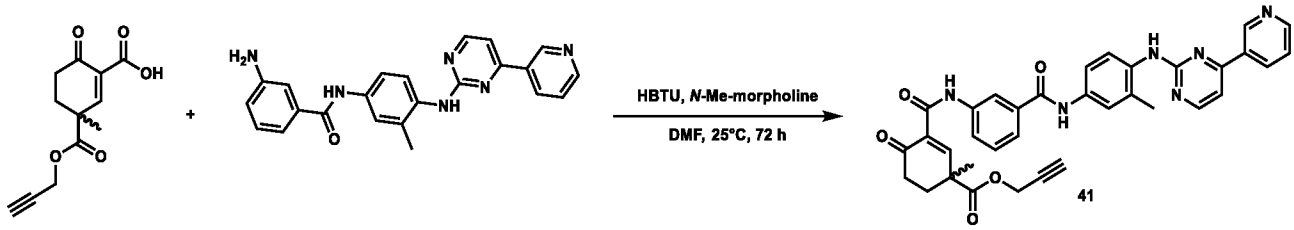
40

Trifluoroacetic acid (159 µL, 2.06 mmol, 3 eq.) was added to a solution of compound **RU83/RU188** (201 mg, 0.69 mmol, 1 eq.) in methylene chloride (5 mL) and then the mixture was stirred at 25°C. The reaction was monitored by TLC and when it was completed (3 h) the reaction mixture was evaporated. The remaining trifluoroacetic acid was removed by redissolving the mixture in toluene and evaporating to dryness at 50°C under reduced pressure which was repeated three times. Compound **5'** was obtained as a yellow oil in quantitative yield (162 mg) and was used in the next step without further purification.

LC-MS ESI $[M+H^+]^+$= 237;

^{1}H NMR (500 MHz, $CDCl_3$): 8.31 (s, 1H), 4.80 – 4.77 (m, 2H), 2.83 – 2.66 (m, 2H), 2.59 (dddd, *J* = 13.1, 6.6, 5.4, 1.2 Hz, 1H), 2.54 (t, *J* = 2.5 Hz, 1H), 2.10 (ddd, *J* = 14.5, 9.9, 5.2 Hz, 1H), 1.60 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$): 201.9, 171.5, 164.5, 163.3, 151.2, 76.7, 76.1, 53.5, 45.3, 34.5, 31.7, 24.3.

Prop-2-yn-1-yl 1-methyl-3-((3-((3-methyl-4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)carbamoyl)phenyl)carbamoyl)-4-oxocyclohex-2-ene-1-carboxylate: (RU-144-IN8 and RU-145-IN-8)

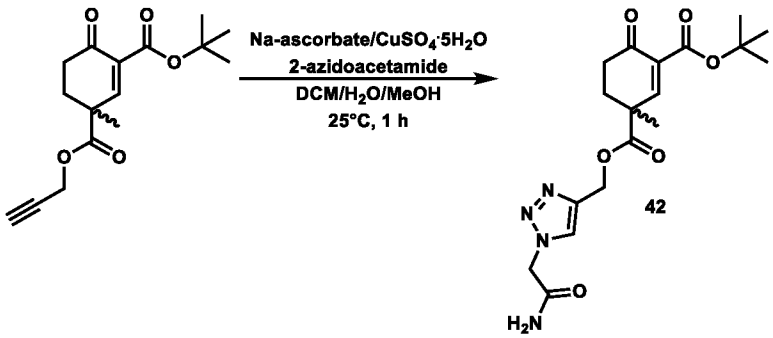


Compound **40** (39.3 mg, 0.166 mmol, 2 eq.) was dissolved in anhydrous dimethylformamide (0.400 mL) in a flame-dried vial under a nitrogen atmosphere, HBTU (63.1 mg, 0.166 mmol, 2 eq.) and *N*-methylmorpholine (37 µL, 0.333 mmol, 4 eq.) were added and the mixture was stirred at 25°C. After 30 minutes a DMF solution (0.400 mL) of compound **20b** (33.0 mg, 0.083 mmol, 1 eq.) was added and the reaction mixture was stirred at 25°C for 72 hours. The reaction was monitored by HPLC-MS and when the starting material was consumed the reaction mixture was diluted with ethyl acetate, washed two times with a 5% citric acid solution, two times with a saturated aqueous Na_2CO_3 solution and two times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The resulting crude product was purified by preparative RP-HPLC using a gradient method on a Gemini® 5µm C18 110 Å column (H_2O:MeCN = 95:5 (0.1 % HCOOH) to 100% MeCN (0.1 % HCOOH)). Compound **RU144/RU145-IN-8** was obtained as a yellow solid (23.0 mg, 45%).

LC-MS ESI $[M+H^+]^+$= 615;

^{1}H NMR (500 MHz, $CDCl_3$): 10.84 (s, 1H), 9.24 (s, 1H), 8.72 (s, 1H), 8.47 (d, *J* = 5.1 Hz, 1H), 8.35 (d, *J* = 7.9 Hz, 1H), 8.25 (s, 1H), 8.20 (s, 1H), 8.10 (s, 1H), 7.98 (d, *J* = 8.6 Hz, 1H), 7.80 (d, *J* = 8.1 Hz, 1H), 7.67 (d, *J* = 7.9 Hz, 1H), 7.62 (d, *J* = 2.3 Hz, 1H), 7.52 (d, *J* = 8.4 Hz, 1H), 7.43 (t, *J* = 7.9 Hz, 1H), 7.14 (d, *J* = 5.0 Hz, 1H), 7.04 (s, 1H), 4.75 (t, *J* = 2.8 Hz, 2H), 2.75 – 2.62 (m, 2H), 2.61 – 2.52 (m, 1H), 2.35 (s, 3H), 2.06 (td, *J* = 8.9, 4.3 Hz, 1H), 1.57 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$) partial: 199.5, 172.2, 165.3, 162.7, 161.3, 161.2, 160.7, 159.2, 151.5, 148.5, 138.3, 136.1, 134.7, 134.1, 134.0, 130.4, 129.6, 129.3, 123.7, 123.6, 123.1, 122.7, 118.8, 118.7, 108.1, 75.9, 53.6, 53.4, 45.2, 35.9, 32.0, 24.5, 18.4.

3-((1-(2-Amino-2-oxoethyl)-1*H*-1,2,3-triazol-4-yl)methyl) 1-(tert-butyl) 3-methyl-6-oxocyclohex-1-ene-1,3-dicarboxylate: (42)

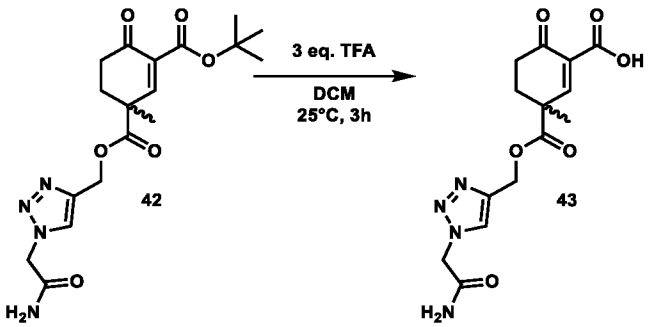


To a solution of compound **40** (80.0 mg, 0.274mmol, 1 eq.) and 2-azidoacetmide (41.1 mg, 0.410 mmol, 1.5 eq.) in methylene chloride (0.5 mL) was added a solution of sodium-ascorbate (27.1 mg, 0.137 mmol, 0.5 eq.) and copper(II) sulfate pentahydrate (6.80 mg, 0.027 mmol, 0.1 eq.) in water (0.5 mL) and methanol (0.25 mL). The reaction mixture was stirred vigorously at 25°C for 1 hour. When the starting material was consumed, the reaction mixture was diluted with 5 mL methylene chloride and washed with water two times. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel using methylene chloride: methanol = 9:1 as eluent. Compound **42** was isolated as a yellow solid (53.1 mg, 50%).

LC-MS ESI $[M+H^+]^+$= 393;

^{1}H NMR (500 MHz, $CDCl_3$): 7.78 (s, 1H), 7.30 (s, 1H), 5.32 (s, 2H), 5.08 (s, 2H), 2.52 – 2.47 (m, 2H), 2.46 – 2.40 (m, 1H), 2.02-1.93 (m, 1H), 1.51 (s, 9H), 1.47 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$): 193.9, 173.2, 167.6, 163.7, 153.9, 133.3, 125.8, 82.5, 58.6, 53.5, 52.2, 44.2, 35.2, 32.0, 28.1, 24.4.

3-(((1-(2-Amino-2-oxoethyl)-1*H*-1,2,3-triazol-4-yl)methoxy)carbonyl)-3-methyl-6-oxocyclohex-1-ene-1-carboxylic acid: (43)

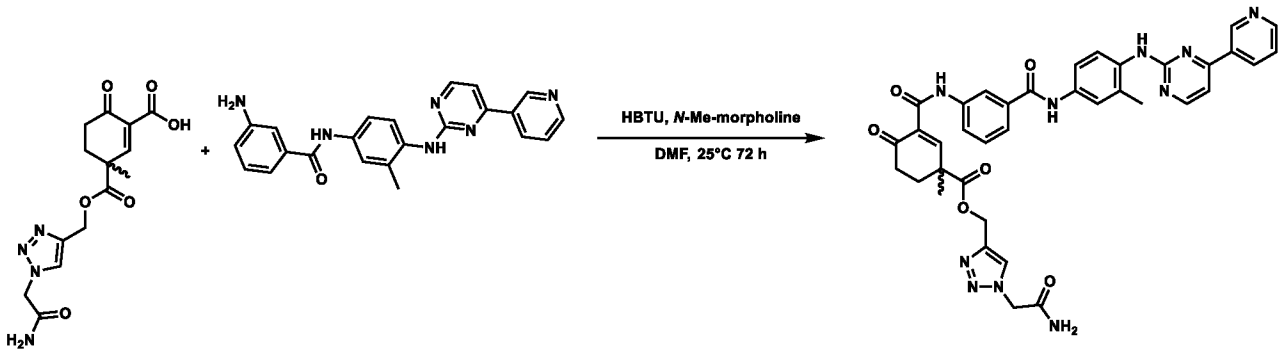


To a solution of compound **42** (53.1 mg, 0.135 mmol, 1 eq.) in methylene chloride (0.65 mL) was added TFA (31 µL, 0.406 mmol, 3 eq.) and the reaction mixture was stirred at 25°C for 3 hours. The reaction was monitored by TLC and when it was completed the reaction mixture was evaporated. The remaining trifluoroacetic acid was removed by redissolving the mixture in toluene and evaporating at 50°C for three times. Compound **43** was obtained as a yellow oil in quantitative yield (45.5 mg) and was used in the next step without further purification.

LC-MS ESI $[M+H^+]^+$= 393;

^{1}H NMR (500 MHz, $CDCl_3$): 8.02 (s, 1H), 7.82 (s, 1H), 5.22 (s, 2H), 5.03 (s, 2H), 2.58 (dt, *J* = 12.0, 5.8 Hz, 2H), 2.47 – 2.36 (m, 1H), 1.97 (ddd, *J* = 14.3, 9.1, 5.5 Hz, 1H), 1.44 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$): 200.7, 172.2, 167.9, 164.2, 163.4, 142.1, 126.8, 125.9, 58.7, 51.9, 45.1, 34.4, 31.5, 23.9.

(1-(2-Amino-2-oxoethyl)-1*H*-1,2,3-triazol-4-yl)methyl 1-methyl-3-((3-((3-methyl-4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)carbamoyl)phenyl)carbamoyl)-4-oxocyclohex-2-ene-1-carboxylate: (RU160-IN-8 and RU161-IN-8)

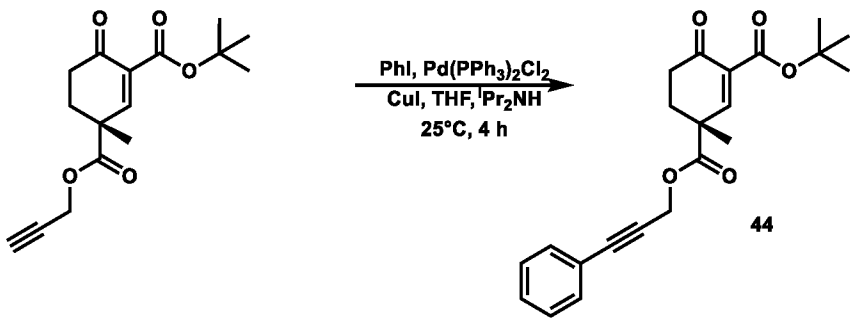


Compound **43** (45.2 mg, 0.134 mmol, 1.5 eq.) was dissolved in anhydrous dimethylformamide (0.550 mL) in a flame-dried vial under a nitrogen atmosphere, HBTU (50.9 mg, 0.134 mmol, 1.5 eq.) and *N*-methylmorpholine (39 µL, 0.358 mmol, 4 eq.) were added and the mixture was stirred at 25°C. After 30 minutes a DMF solution (0.550 mL) of compound **20b** (35.5 mg, 0.089 mmol, 1 eq.) was added and the reaction mixture was stirred at 25°C for 72 hours. The reaction was monitored by HPLC-MS and when the starting material was consumed the reaction mixture was diluted with ethyl acetate, washed two times with a 5% citric acid solution, two times with a saturated aqueous Na_2CO_3 solution and two times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The resulting crude product was purified by preparative RP-HPLC using a gradient method on a Gemini® 5µm C18 110 Å column (H_2O:MeCN = 95:5 (0.1 % HCOOH) to 100% MeCN (0.1 % HCOOH)). Compound **RU160/RU161-IN-8** was obtained as a yellow solid (19.3 mg, 30%).

LC-MS ESI $[M+H^+]^+$= 715;

^{1}H NMR (500 MHz, $CDCl_3$): 10.78 (s, 1H), 9.13 (s, 1H), 8.61 (s, 1H), 8.39 (d, *J* = 5.2 Hz, 1H), 8.31 (d, *J* = 8.0 Hz, 1H), 8.13 (s, 1H), 8.11 (d, *J* = 2.8 Hz, 1H), 7.84 – 7.78 (m, 2H), 7.65 (d, *J* = 7.7 Hz, 1H), 7.59 (s, 1H), 7.51 (t, *J* = 8.6 Hz, 2H), 7.39 (t, *J* = 7.9 Hz, 1H), 7.09 (d, *J* = 5.2 Hz, 1H), 5.25 (s, 2H), 5.00 (s, 2H), 2.59 (ddd, *J* = 14.8, 11.4, 6.7 Hz, 2H), 2.45 (dt, *J* = 12.9, 6.1 Hz, 1H), 2.29 (s, 3H), 1.98 (ddd, *J* = 14.3, 9.4, 6.1 Hz, 1H), 1.48 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$): 199.3, 172.7, 167.6, 166.2, 162.5, 161.5, 161.0, 160.9, 159.0, 150.8, 148.0, 142.3, 137.3, 136.1, 135.2, 134.7, 133.6, 132.0, 129.4, 129.1, 125.8, 125.0, 124.4, 123.9, 123.5, 122.9, 121.5, 119.4, 118.9, 107.8, 58.7, 45.1, 35.6, 31.8, 24.2, 18.2, 18.0.

1-(tert-butyl) 3-(3-phenylprop-2-yn-1-yl) (R)-3-methyl-6-oxocyclohex-1-ene-1,3-dicarboxylate: (44)

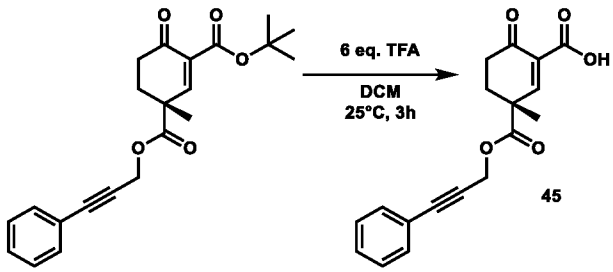


Into a flame-dried vial under a nitrogen atmosphere was added $Pd(PPh_3)_2Cl_2$ (7.2 mg, 0.010 mmol, 0.06 eq.) and CuI (3.6 mg, 0.019 mmol, 0.11 eq.). To this vial was added a solution of compound ***R*-40** (50.0 mg, 0.171 mmol, 1 eq.) and iodobenzene (34.9 mg, 0.171 mmol, 1 eq.) in anhydrous tetrahydrofuran (0.375 mL) and diisopropylamine (0.375 mL). The reaction mixture was stirred at 25°C for 4 hours and when the starting material was consumed it was filtered through a pad of Celite then evaporated. The residue was purified by flash column chromatography on silica gel using hexane: ethyl acetate = 1:1 as eluent to afford compound **44** as a yellow oil (31.7 mg, 50%).

LC-MS ESI $[M+H^+]^+$= 369;

^{1}H NMR (500 MHz, $CDCl_3$): 7.45 (d, *J* = 1.6 Hz, 1H), 7.43 (d, *J* = 1.9 Hz, 1H), 7.39 (s, 1H), 7.35 – 7.33 (m, 2H), 7.32 – 7.29 (m, 1H), 5.02 (d, *J* = 15.5 Hz, 1H), 4.97 (d, *J* = 15.5 Hz, 1H)2.65 – 2.55 (m, 1H), 2.55 – 2.47 (m, 2H), 2.09 – 1.98 (m, 1H), 1.53 (s, 3H), 1.52 (s, 9H).
^{13}C NMR (125 MHz, $CDCl_3$): 193.5, 172.8, 163.7, 153.5, 133.7, 132.0, 129.1, 128.5, 122.0, 87.2, 82.3, 82.3, 54.1, 44.3, 35.4, 32.1, 28.2, 24.5.

(R)-3-methyl-6-oxo-3-(((3-phenylprop-2-yn-1-yl)oxy)carbonyl)cyclohex-1-ene-1-carboxylic acid: (45)

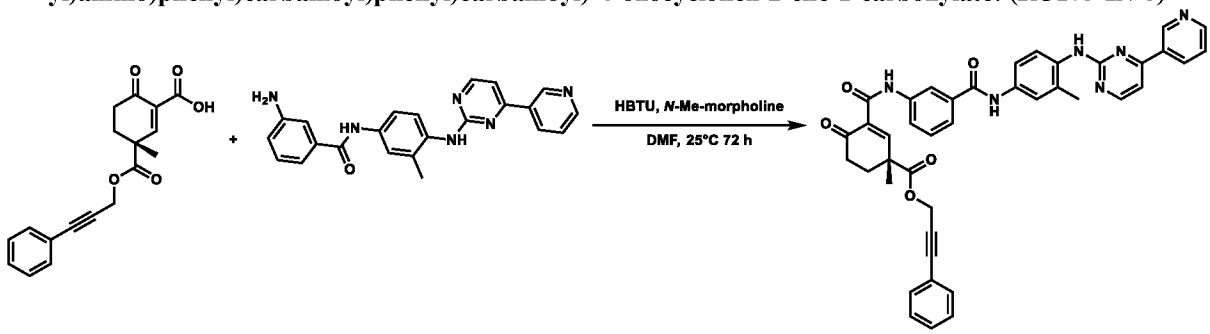


To a solution of compound **44** (31.7 mg, 0.086 mmol, 1 eq.) in methylene chloride (0.80 mL) was added TFA (40 µL, 0.516 mmol, 6 eq.) and the reaction mixture was stirred at 25°C for 3 hours. The reaction was monitored by TLC and when it was completed the reaction mixture was evaporated. The remaining trifluoroacetic acid was removed by redissolving the mixture in toluene and evaporating to dryness under reduced pressure at 50°C which was repeated three times. Compound **45** was obtained as a brown oil (25.3 mg, 94%).

LC-MS ESI $[M+H^+]^+$= 313;

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.31 (s, 1H), 7.48 – 7.39 (m, 2H), 7.37 – 7.29 (m, 2H), 7.35 – 7.28 (m, 1H), 4.98 (d, *J* = 2.7 Hz, 2H), 2.82 – 2.66 (m, 2H), 2.68 – 2.51 (m, 1H), 2.08 (m, 1H), 1.59 (d, *J* = 0.8 Hz, 3H).
^{13}C NMR (125 MHz, $CDCl_3$): 202.0, 171.6, 164.8, 163.4, 132.0, 129.2, 128.5, 126.2, 121.8, 87.6, 81.9, 54.5, 45.4, 34.6, 31.8, 24.4.

3-phenylprop-2-yn-1-yl (*R*)-1-meth67utanone3-((3-methyl-4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)carbamoyl)phenyl)carbamoyl)-4-oxocyclohex-2-ene-1-carboxylate: (RU173-IN-8)



Compound **45** (25.3 mg, 0.081 mmol, 1.25 eq.) was dissolved in anhydrous dimethylformamide (0.350 mL) in a flame-dried vial under a nitrogen atmosphere, HBTU (30.7 mg, 0.081 mmol, 1.25 eq.) and *N*-methylmorpholine (18 µL, 0.162 mmol, 2.5 eq.) were added and the mixture was stirred at 25°C. After 30 minutes a DMF solution (0.350 mL) of compound **20b** (25.7 mg, 0.065 mmol, 1 eq.) was added and the reaction mixture was stirred at 25°C for 72 hours. The reaction was monitored by HPLC-MS and when the starting material was consumed the reaction mixture was diluted with ethyl acetate, washed two times with a 5% citric acid solution, two times with a saturated aqueous Na_2CO_3 solution and two times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The resulting crude product was purified by preparative RP-HPLC using a gradient method on a Gemini® 5µm C18 110 Å column (H_2O:MeCN = 95:5 (0.1 % HCOOH) to 100% MeCN (0.1 % HCOOH)). Compound **RU173-IN-8** was obtained as a yellow solid (10.9 mg, 24%).

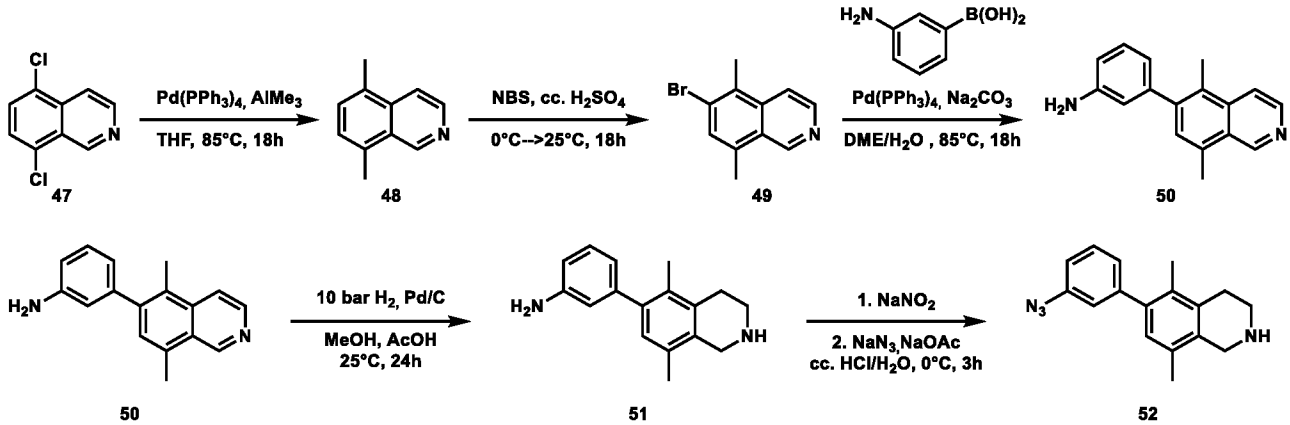
LC-MS ESI $[M+H^+]^+$= 691;

^{1}H NMR (500 MHz, $CDCl_3$): 9.22 (s, 1H), 8.63 (s, 1H), 8.46 (d, *J* = 8.0 Hz, 1H), 8.42 (d, *J* = 5.2 Hz, 1H), 8.16 (s, 1H), 8.15-8.13 (m, 1H), 7.85 – 7.7 (m, 1H), 7.70 (dd, *J* = 7.7, 1.5 Hz, 1H), 7.64 – 7.62 (m, 2H), 7.57 (dd, *J* = 8.5, 2.4 Hz, 1H), 7.57 – 7.49 (m, 2H), 7.55-7.50 (m, 1H), 7.48 (t, *J* = 7.9 Hz, 1H), 7.42 – 7.41 (m, 1H), 7.40-7.39 (m, 1H), 7.34 – 7.30 (m, 4H), 7.28 (d, *J* = 5.3 Hz, 1H), 5.03 (s, 2H), 2.57 – 2.44 (m, 2H), 2.17 – 2.09 (m, 1H), 2.06 – 2.01 (m, 1H), 1.59 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$): 200.1, 173.7, 168.0, 163.5, 162.7, 162.6, 161.4, 160.1, 153.5, 151.6, 148.9, 139.1, 137.2, 136.5, 136.4, 135.2, 133.9, 132.7, 132.6, 130.9, 130.2, 129.9, 129.8, 129.4, 126.1, 124.8, 124.1, 123.1, 120.7, 120.1, 108.4, 87.7, 83.4, 54.8, 46.1, 36.3, 32.8, 24.4, 18.5.

4-azido-*N*,*N*-dimethylaniline: (46)

4-azido-*N*,*N*-dimethylaniline was synthesized according to the literature procedure (Schulz et al., 2019)

6-(3-azidophenyl)-5,8-dimethyl-1,2,3,4-tetrahydroisoquinoline: (52)



To a solution of compound **47** (1.20 g, 6.00 mmol, 1 eq.) in 14 mL anhydrous THF in a pressure vial was added $Pd(PPh_3)_4$ (300.0 mg, 0.26 mmol, 0.04 eq.) under a N_2 atmosphere. A 2 M toluene solution of $AlMe_3$ (11mL, 22.0 mmol, 3.7 eq.) was added dropwise then the reaction mixture was stirred at 85°C for 18 hours. After the reaction was complete, the mixture was cooled to room temperature and poured into ice-water. The aqueous phase was extracted three times with methylene chloride. The combined organic phase was then washed with water and brine. Afterward, it was dried over anhydrous Na_2SO_4, filtered, and the solvent was evaporated under reduced pressure The crude product was purified by flash column chromatography giving compound **48** as a brown oil (0.43g, 45%).

^1H NMR (500 MHz, $CDCl_3$): 9.43-9.42 (m, 1H), 8.58-8.56 (m, 1H), 7.71-7.70 (m, 1H, 7.38-7.34 (m, 1H), 7.25-7.22 (m, 1H), 2.72 (s, 3H), 2.59 (s, 3H);

A round bottom flask was charged with 8.5 mL of H_2SO_4. After cooling to 0°C, compound **48** (0.833 g, 5.30 mmol, 1 equiv.) was added, followed by NBS (1.13 g, 5.3 mmol, 1.18 equiv.) in small portions. The reaction mixture was allowed to warm to 25°C and stirred for 18 hours. After completion, the reaction mixture was poured into ice-water and the pH was adjusted to 14 with a 25% NH_3 solution. The aqueous phase was extracted three times with ethyl acetate. The combined organic phase was washed with water and brine, dried over anhydrous Na_2SO_4, filtered, and the solvent was evaporated under reduced pressure. The crude product was purified by recrystallization, yielding compound **49** as brown crystals (0.770 g, 62%).

^1H NMR (500 MHz, $CDCl_3$): 9.35-9.33 (m, 1H), 8.52-8.50 (m, 1H), 7.58-7.56 (m, 1H), 7.5-7.49 (m, 1H), 2.69 (s, 3H), 2.47 (s, 3H);

A pressure vessel was charged with compound **49** (0.750 g, 3.19 mmol 1 eq.), (3-aminophenyl)boronic acid (0.655 g, 4.78 mmol, 1.5 eq.), Na_2CO_3 (0.676 g, 6.38 mmol, 2 eq.), $Pd(PPh_3)_4$ (0.220 g, 0.19 mmol, 0.059 eq.), 9.6 mL dimethoxyethane and 4.8 mL water and the reaction mixture was stirred at 85°C for 18 hours under a N_2 atmosphere. After completion the reaction mixture was cooled to 25°C and water and ethyl acetate was added to it. After phase separation the aqueous phase was extracted 3 times with ethyl acetate. The combined organic phase was washed with water and brine, then it was dried over anhydrous Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The crude product was purified by flash column chromatography giving compound **50** as brownish crystals (0.680 g, 86%).

^1H NMR (500 MHz, $CDCl_3$): 9.52 (s, 1H), 8.58 (d, J = 7.5 Hz, 1H), 7.75 – 7.72 (m, 1H), 7.40 (s, 1H), 7.21 (td, J = 7.7, 1.9 Hz, 1H), 6.72 – 6.67 (m, 2H), 6.65 – 6.63 (m, 1H), 2.64 (s, 3H), 2.61 (s, 3H);

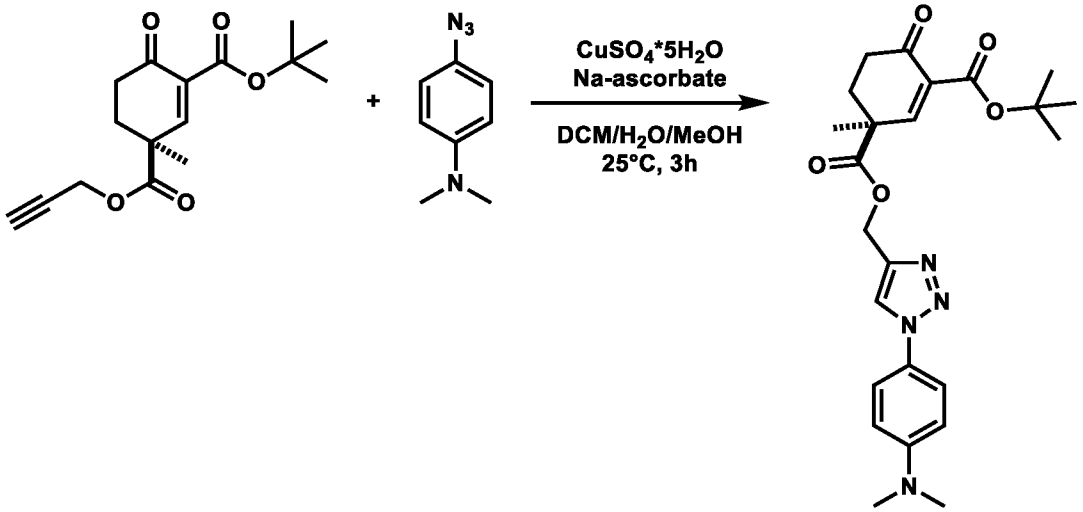
A pressure vessel was charged with a solution of compound **50** (0.400 g, 1.61 mmol, 1 eq.) in 8 mL methanol and 94 µL 98% acetic acid. The atmosphere was changed to N_2 then palladium on carbon was added (0.171 g, 0.161 mmol, 0.1 eq.) and vessel was charged with 10 bar H_2 and the reaction mixture was stirred at 25°C for 24 hours. After completion the reaction mixture was filtered on a pad of Celite then evaporated. The crude mixture was dissolved in methylene chloride then washed 2 times with a 10% NaOH solution. The organic phase was washed with water and brine, then it was dried over anhydrous Na_2SO_4, filtered and the solvent was evaporated under reduced pressure giving compound **51** as an orange oil (0.230g, 56%).

LC-MS ESI $[M+H^+]^+$= 253;

^1H NMR (500 MHz, $CDCl_3$): 7.12 – 7.06 (m, 2H), 6.97 (s, 1H), 6.75 – 6.70 (m, 2H), 3.99 (s, 2H), 3.18 (t, J = 6.0 Hz, 2H), 2.71 (t, J = 6.0 Hz, 2H), 2.23 (s, 3H), 2.08 (s, 3H);

A solution of compound **51** (0.320 g, 1.26 mmol, 1 eq.) in 6 mL HCl was cooled to 0 ° C, then a solution of $NaNO_2$ (0.200 g, 2.9 mmol, 2.3 eq.) in 6 mL water was added dropwise, and the reaction mixture was stirred for 2 hours at 0 ° C. After this, a solution of NaN_3 (0.188g, 2.9 mmol, 2.3 eq.) and NaOAc (2.62g, 3.19 mmol, 2.5 eq.) in 6 mL water was added dropwise, and the reaction mixture was stirred for a further 1 hour at 0 ° C. The pH was set to 7 with an aqueous NaHCO3 solution, and the aqueous phase was extracted 3 times with chloroform. The combined organic phase was washed with water and brine, then it was dried over anhydrous Na_2SO_4, filtered, and the solvent was evaporated under reduced pressure, giving compound **52** as a white solid without further purification (0.110 g, 67%).

1-(*tert*-butyl) 3-((1-(4-(dimethylamino)phenyl)-1*H*-1,2,3-triazol-4-yl)methyl) 3-methyl-6-oxocyclohex-1-ene-1,3-dicarboxylate
(RU103/RU189)

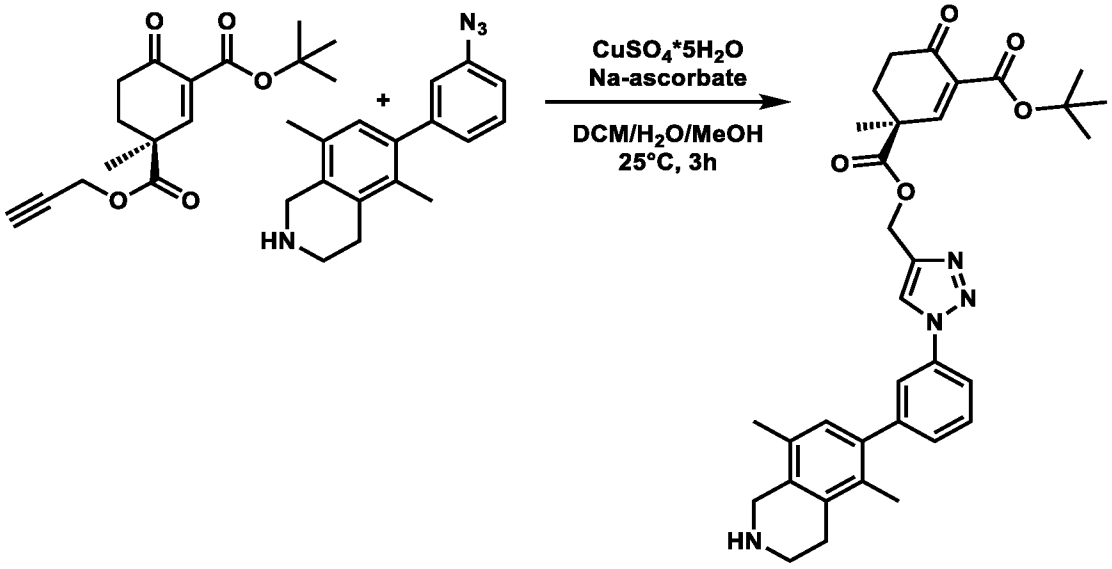


To the solution of **RU83/RU188** (30.0 mg, 0.103 mmol, 1 eq.) in methylene chloride (0.2 mL) was added 4-azido-N,N-dimethylaniline (16.2 mg, 0.103 mmol, 1 eq.). To this mixture was added the solution of copper(II) sulfate pentahydrate (2.6 mg, 0.010 mmol, 0.1 eq.) and sodium ascorbate (10.2 mg, 0.051 mmol, 0.5 eq.) in water (0.2 mL) and methanol (0.1 mL). The reaction mixture was vigorously stirred at 25°C. After 3 hours the mixture was diluted with methylene chloride and water then the aqueous phase was washed with methylene chloride two times. The combined organic phases were washed with brine then dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure. The crude product was purified by preparative RP-HPLC using a gradient method on a Gemini® 5µm C18 110 Å column (H_2O:MeCN = 95:5 (0.1 % HCOOH) to 100% MeCN (0.1 % HCOOH)). Compound **RU103/RU189** was obtained as a yellow solid (36.1 mg, 77%).

LC-MS ESI $[M-(^tBu)+H^+]^+$= 399

^1H NMR (500 MHz, $CDCl_3$): 7.89 (s, 1H), 7.52 (d, J = 9.0 Hz, 2H), 7.34 (s, 1H), 6.75 (d, *J* = 9.1 Hz, 2H), 5.35 (q, J = 12.7 Hz, 2H), 3.00 (s, 6H), 2.56 – 2.47 (m, 2H), 2.46 – 2.40 (m, 1H), 2.03 – 1.94 (m, 1H), 1.49 (s, 9H), 1.47 (s, 3H).
^{13}C NMR (125 MHz, $CDCl_3$): 193.5, 173.4, 163.7, 153.6, 150.9, 133.6, 126.5, 122.1, 122.0, 112.4, 82.3, 59.0, 44.3, 40.5, 35.3, 32.0, 28.1 (q), 24.5, 24.4;

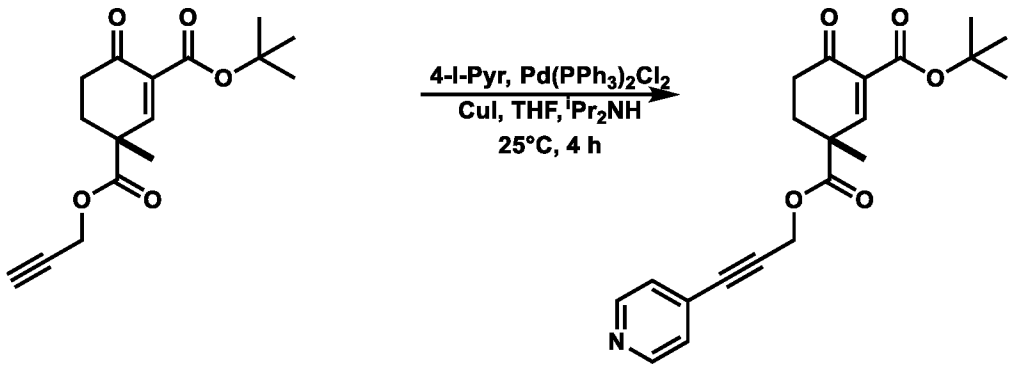
1-(*tert*-butyl) 3-((1-(3-(5,8-dimethyl-1,2,3,4-tetrahydroisoquinolin-6-yl)phenyl)-1H-1,2,3-triazol-4-yl)methyl) (*S*)-3-methyl-6-oxocyclohex-1-ene-1,3-dicarboxylate
(RU115)



To the solution of **RU83** (100.0 mg, 0.340 mmol, 1 eq.) in *tert*-butyl alcohol (1.0 mL) was added 6-(3-azidophenyl)-5,8-dimethyl-1,2,3,4-tetrahydroisoquinoline (95.0 mg, 0.340 mmol, 1 eq.). To this mixture was added the solution of copper(II) sulfate pentahydrate (16.0 mg, 0.068 mmol, 0.2 eq.) and sodium ascorbate (33.0 mg, 0.170 mmol, 0.5 eq.) in water (1.0 mL). The reaction mixture was vigorously stirred at 25°C. After 3 days the mixture was diluted with methylene chloride and water then the aqueous phase was washed with methylene chloride two times. The combined organic phases were washed with brine then dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure. **RU115** obtained as a green oil and was used without further purification (160.0 mg, 83%).

LC-MS ESI $[M+H^+]^+$= 571

1-(*tert*-butyl) 3-(3-(pyridin-4-yl)prop-2-yn-1-yl) 3-methyl-6-oxocyclohex-1-ene-1,3-dicarboxylate (RU140/RU141)

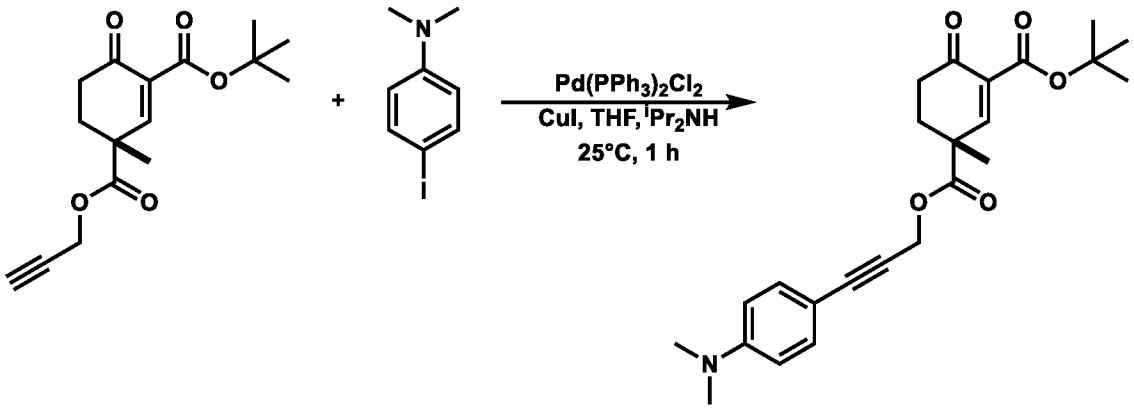


Into a flame-dried vial under a nitrogen atmosphere was added $Pd(PPh_3)_2Cl_2$ (7.2 mg, 0.010 mmol, 0.06 eq.) and CuI (3.6 mg, 0.019 mmol, 0.11 eq.). To this vial was added a solution of compound **RU83/RU188** (50.0 mg, 0.171 mmol, 1 eq.) and 4-iodopyridine (35.1 mg, 0.171 mmol, 1 eq.) in anhydrous tetrahydrofuran (0.375 mL) and diisopropylamine (0.375 mL). The reaction mixture was stirred at 25°C for 4 hours and when the starting material was consumed it was filtered through a pad of Celite then evaporated. The residue was purified by flash column chromatography on silica gel using hexane: ethyl acetate = 1:1 as eluent to afford **RU140/141** as yellow oils (39.1 mg, 62%).

LC-MS ESI $[M+H^+]^+$= 370

^1H NMR (500 MHz, $CDCl_3$): 8.59 (d, J = 5.0 Hz, 2H), 7.37 (s, 1H), 7.30 – 7.28 (m, 2H), 5.04 – 4.90 (m, 2H), 2.64 – 2.40 (m, 3H), 2.10 – 1.97 (m, 1H), 1.54 (s, 3H), 1.52 (s, 9H).

1-(*tert*-butyl) 3-(3-(4-(dimethylamino)phenyl)prop-2-yn-1-yl) 3-methyl-6-oxocyclohex-1-ene-1,3-dicarboxylate (RU214/RU215)

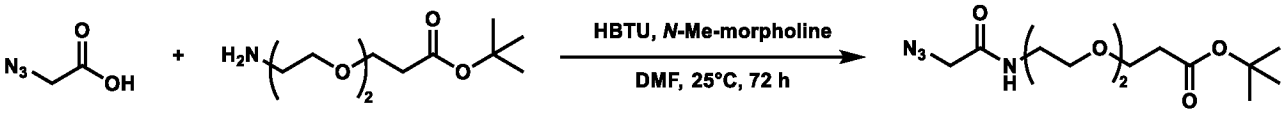


Into a flame-dried vial under a nitrogen atmosphere was added $Pd(PPh_3)_2Cl_2$ (14.4 mg, 0.020 mmol, 0.06 eq.) and CuI (7.2 mg, 0.038 mmol, 0.11 eq.). To this vial was added a solution of compound **RU83/RU188** (100.0 mg, 0.171 mmol, 1 eq.) and 4-iodo-*N*,*N*-dimethylaniline (84.5 mg, 0.342 mmol, 1 eq.) in anhydrous tetrahydrofuran (1.0 mL) and diisopropylamine (1.0 mL). The reaction mixture was stirred at 25°C for 1 hour and when the starting material was consumed it was filtered through a pad of Celite then evaporated. The residue was purified by flash column chromatography on silica gel using hexane: ethyl acetate = 1:1 as eluent to afford **RU214/215** as yellow oils (40.2 mg, 29%).

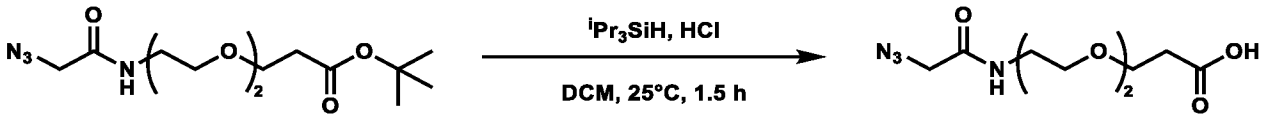
LC-MS ESI $[M+H^+]^+$= 356

^{1}H NMR (500 MHz, $CDCl_3$): 7.40 (s, 1H), 7.32 (d, *J* = 8.9 Hz, 2H), 6.61 (d, *J* = 8.9 Hz, 2H), 4.97 (q, *J* = 15.4 Hz, 2H), 2.98 (s, 6H), 2.63 – 2.56 (m, 1H), 2.55 – 2.46 (m, 2H), 2.03 – 1.99 (m, 1H), 1.54 (s, 3H), 1.52 (s, 9H).
^{13}C NMR (125 MHz, $CDCl_3$): 193.6, 172.9, 163.7, 153.7, 150.6, 133.2, 130.3, 111.8, 108.6, 88.5, 82.3, 80.2, 54.6, 44.3, 40.2, 35.4, 32.0, 28.2, 24.5.

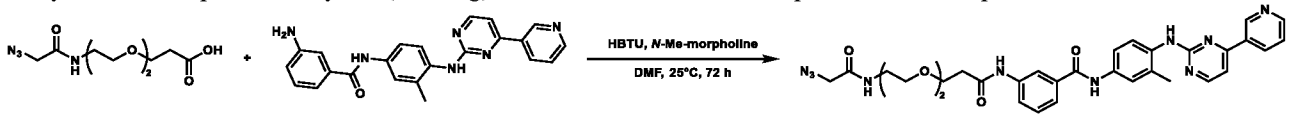
3-(3-(2-(2-(2-azidoacetamido)ethoxy)ethoxy)propanamido)-*N*-(3-methyl-4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)benzamide



2-azidoacetic acid (68.0 mg, 0.670 mmol, 1.8 eq.) was dissolved in anhydrous dimethylformamide (1.0 mL) in a flame-dried vial under a nitrogen atmosphere, HBTU (260.0 mg, 0.670 mmol, 1.8 eq.) and *N*-methylmorpholine (120 µL, 1.10 mmol, 3.0 eq.) were added and the mixture was stirred at 25°C. After 30 minutes an anhydrous DMF solution (1.0 mL) of *tert*-butyl 3-(2-(2-aminoethoxy)ethoxy)propanoate (88.0 mg, 0.380 mmol, 1 eq.) was added and the reaction mixture was stirred at 25°C for 3 days. The reaction was monitored by HPLC-MS and when the starting material was consumed the reaction mixture was diluted with ethyl acetate, washed two times with a 5% citric acid solution, two times with a saturated aqueous Na_2CO_3 solution, and two times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The resulting yellow oil crude *tert*-butyl 3-(2-(2-(2-azidoacetamido)ethoxy)ethoxy)propanoate was used without further purification (114.0 mg, 96%).



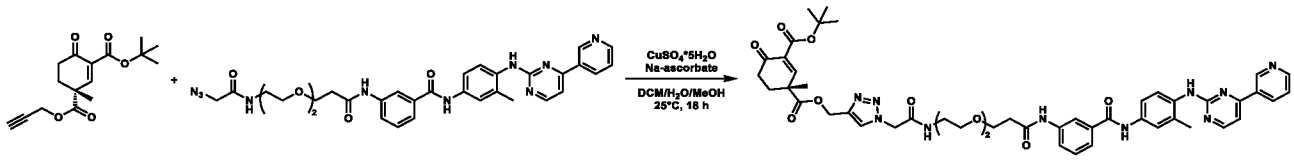
To a solution of *tert*-butyl 3-(2-(2-(2-azidoacetamido)ethoxy)ethoxy)propanoate (94.9 mg, 0.300 mmol, 1 eq.) in methylene chloride (5.0 mL), was added triisopropyl silane (61.5 µL, 0.300 mmol, 1 eq.) and a 37% HCl solution (120.0 µL, 1.50 mmol, 5 eq.) and the reaction mixture was stirred vigorously at 25°C for 1 hour. When it was completed the reaction mixture was evaporated. The remaining trifluoroacetic acid was removed by redissolving the mixture in toluene and evaporating to dryness at 50°C three times. 3-(2-(2-(2-azidoacetamido)ethoxy)ethoxy)propanoic acid was obtained as a yellow oil in quantitative yield (89.0 mg) and was used in the next step without further purification.



3-(2-(2-(2-azidoacetamido)ethoxy)ethoxy)propanoic acid (93.0 mg, 0.360 mmol, 1.4 eq.) was dissolved in anhydrous dimethylformamide (3.0 mL) in a flame-dried vial under a nitrogen atmosphere, HBTU (134.0 mg, 0.360 mmol, 1.4 eq.) and *N*-methylmorpholine (100 µL, 0.910 mmol, 3.6 eq.) were added and the mixture was stirred at 25°C. After 30 minutes an anhydrous DMF solution (3.0 mL) of compound **19** (100.0 mg, 0.252 mmol, 1 eq.) was added and the reaction mixture was stirred at 25°C for 3 days. The reaction was monitored by HPLC-MS and when the starting material was consumed the reaction mixture was diluted with ethyl acetate, washed two times with a 5% citric acid solution, two times with a saturated aqueous Na_2CO_3 solution, and two times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The resulting crude 3-(3-(2-(2-(2-azidoacetamido)ethoxy)ethoxy)propanamido)-N-(3-methyl-4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)benzamide was used without further purification.

LC-MS ESI $[M+H^+]^+$= 639

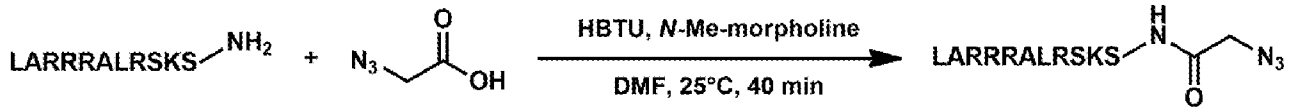
1-(*tert*-butyl) 3-((1-(2-((2-(2-(3-((3-((3-methyl-4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)carbamoyl)phenyl)amino)-3-oxopropoxy)ethoxy)ethyl)amino)-2-oxoethyl)-1*H*-1,2,3-triazol-4-yl)methyl) (*R*)-3-methyl-6-oxocyclohex-1-ene-1,3-dicarboxylate (RU188-PEG-IN-8)



To the solution of **RU188** (95.8 mg, 0.328 mmol, 1.3 eq.) in methylene chloride (0.45 mL) was added 3-(3-(2-(2-(2-azidoacetamido)ethoxy)ethoxy)propanamido)-N-(3-methyl-4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)benzamide (161.0 mg, 0.252 mmol, 1 eq.). To this mixture was added the solution of copper(II) sulfate pentahydrate (6.3 mg, 0.025 mmol, 0.1 eq.) and sodium ascorbate (25.0 mg, 0.126 mmol, 0.5 eq.) in water (0.450 mL) and methanol (0.225 mL). The reaction mixture was vigorously stirred at 25°C. After 18 hours the mixture was diluted with methylene chloride and water then the aqueous phase was washed with methylene chloride two times. The combined organic phases were washed with brine then dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure. The crude product was purified by preparative RP-HPLC using a gradient method on a Gemini® 5µm C18 110 Å column (H_2O:MeCN = 95:5 (0.1 % HCOOH) to 100% MeCN (0.1 % HCOOH)). Compound **RU188-PEG-IN-8** was obtained as a yellow solid.

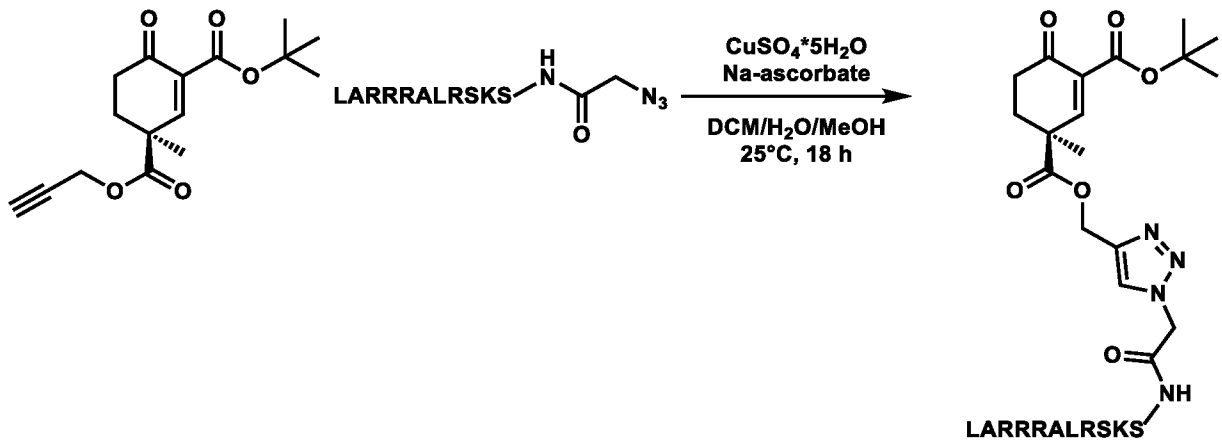
LC-MS ESI $[M+H^+]^+$= 931

Azido pepMNK1



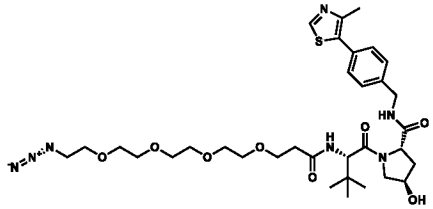
The azidoacetic acid was synthesized according to literature procedure (Schmitz et al, 2016). The peptide was chemically synthesized on an automated PSE Peptide Synthesizer (Protein Technologies, Tucson) with standard Fmoc/^{t}Bu strategy. After solid phase peptide synthesis and subsequent cleavage from resin, the peptides were isolated by semi-preparative RP-HPLC using water with 0.1% formic acid as eluent A and acetonitrile with 0.1% formic acid as eluent B.
LC-MS ESI $[M+H^+]^+$= 1397

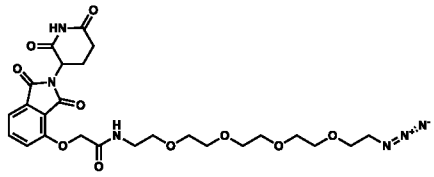
RU83-click-pepMNK1_C



To the solution of **RU83** (20.0 mg, 0.068 mmol, 1 eq.) in *tert*-butyl alcohol (0.15 mL) was added azido-pepMNK1 (96.0 mg, 0.068 mmol, 1 eq.). To this mixture was added the solution of copper(II) sulfate pentahydrate (17.0 mg, 0.068 mmol, 1 eq.) and sodium ascorbate (14.0 mg, 0.068 mmol, 1 eq.) in water (0.1 mL). The reaction mixture was vigorously stirred at 25°C. After 3 days the mixture was diluted with water then filtered using a syringe filter and the target compound was purified by a semi-preparative HPLC giving the title compound as white solid.

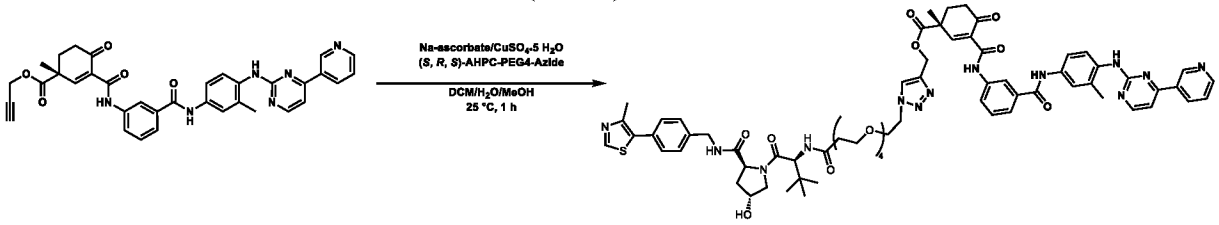
LC-MS ESI $[M+H^+]^+$= 1689





Compound (*S*, *R*, *S*)-AHPC-PEG4-azide and Thalidomide-O-amido-PEG4-azide were purchased from BroadPharm, USA.

Preparation of (1-((*S*)-17-((2S,4*R*)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidine-1-carbonyl)-18,18-dimethyl-15-oxo-3,6,9,12-tetraoxa-16-azanonadecyl)-1*H*-1,2,3-triazol-4-yl)methyl (*R*)-1-methyl-3-((3-((3-methyl-4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)carbamoyl)phenyl)carbamoyl)-4-oxocyclohex-2-ene-1-carboxylate:
(RU219)

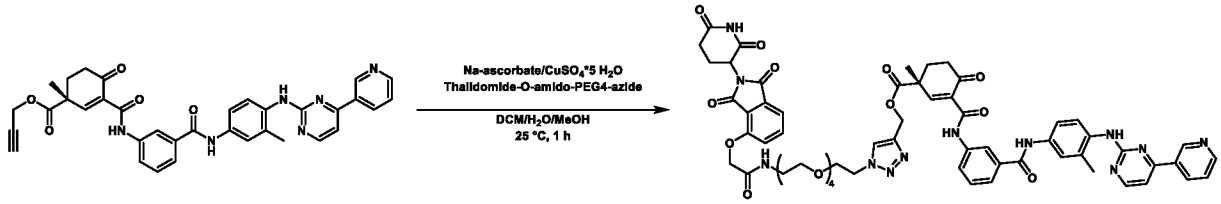


To a solution of compound **RU144** (23.6 mg, 0.038 mmol, 1.5 eq.) and (*S*, *R*, *S*)-AHPC-PEG4-azide (18.0 mg, 0.026 mmol, 1 eq.) in methylene chloride (0.10 mL) was added a solution of sodium-ascorbate (2.5 mg, 0.013 mmol, 0.5 eq.) and copper(II) sulfate pentahydrate (0.6 mg, 0.003 mmol, 0.1 eq.) in water (0.10 mL) and methanol (0.05 mL). The reaction mixture was stirred vigorously at 25°C for 1 hour. When the starting material was consumed, the reaction mixture was diluted with 2 mL methylene chloride and washed with diluted sodium chloride solution two times. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The crude product was purified by preparative RP-HPLC using a gradient method on a Gemini® 5µm C18 110 Å column (H_2O:MeCN = 95:5 (0.1 % HCOOH) to 100% MeCN (0.1 % HCOOH)). Compound **RU219** was isolated as a yellow solid (12.7 mg, 38 %).

LC-MS ESI $[M+H^+]^+$ = 1318;

^{1}H NMR (600 MHz, $CDCl_3$): δ 10.81 (s, 1H), 9.24 (s, 1H), 8.70 (s, 1H), 8.65 (s, 1H), 8.57 (s, 1H), 8.45 (d, J = 5.2 Hz, 1H), 8.35 (d, J = 8.0 Hz, 1H), 8.22 (s, 1H), 8.19 (s, 1H), 7.94 (d, J = 8.7 Hz, 1H), 7.82 (s, 1H), 7.77 – 7.73 (m, 1H), 7.69 – 7.68 (m, 1H), 7.68 – 7.67 (m, 1H), 7.54 (dd, J = 8.6, 2.5 Hz, 1H), 7.45 (s, 1H), 7.42 (t, J = 7.9 Hz, 1H), 7.31 – 7.31 (m, 2H), 7.31 –7.31 (m, 2H), 7.17 (s, 1H), 7.14 (d, J = 5.2 Hz, 1H), 6.95 (d, J = 8.5 Hz, 1H), 5.29 (s, 4H), 4.68 (t, J = 8.0 Hz, 1H), 4.51 – 4.51 (m, 1H), 4.51 (s, 1H), 4.48 (s, 1H), 4.47 – 4.47 (m, 1H), 4.30 (dd, J = 15.1, 5.4 Hz, 1H), 4.01 (d, J = 11.3 Hz, 1H), 3.83 (t, J = 5.0 Hz, 2H), 3.64 (t, J = 5.9 Hz, 2H), 3.58 – 3.57 (m, 1H), 3.57 – 3.54 (m, 12H), 2.71 – 2.57 (m, 2H), 2.49 (s, 1H), 2.48 (s, 3H), 2.43 (s, 1H), 2.40 – 2.39 (m, 2H), 2.35 (s, 3H), 2.10 (dd, J = 13.4, 8.3 Hz, 1H), 2.03 – 2.01 (m, 1H), 1.53 (s, 3H), 0.91 (s, 9H).
^{13}C NMR (151 MHz, $CDCl_3$): δ 199.4, 172.7, 171.7, 171.6, 171.0, 165.4, 162.6, 161.5, 160.8, 160.7, 158.8, 151.1, 150.3, 148.4, 148.2, 141.8, 138.2, 138.0, 136.0, 135.2, 134.8, 134.4, 133.6, 132.8, 131.6, 130.8, 130.6, 129.4, 129.4, 129.2, 128.0, 125.0, 123.8, 123.6, 123.2, 122.6, 119.0, 118.7, 107.9, 70.5, 70.46 –
70.42 (m), 70.39, 70.37, 70.35, 70.0, 69.3, 67.1, 59.0, 58.6, 57.7, 56.7, 50.3, 45.1, 43.1, 36.6, 36.2, 35.7, 35.1, 31.9, 26.4, 24.3, 18.4, 16.0.

Preparation of (1-(1-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)-2-oxo-6,9,12,15-tetraoxa-3-azaheptadecan-17-yl)-1*H*-1,2,3-triazol-4-yl)methyl (1*R*)-1-methyl-3-((3-((3-methyl-4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)carbamoyl)phenyl)carbamoyl)-4-oxocyclohex-2-ene-1-carboxylate:
(RU231)



To a solution of compound **RU144** (32.0 mg, 0.052 mmol, 1.5 eq.) and Thalidomide-O-amido-PEG4-azide (20.0 mg, 0.035 mmol, 1 eq.) in methylene chloride (0.14 mL) was added a solution of sodium-ascorbate (3.4 mg, 0.017 mmol, 0.5 eq.) and copper(II) sulfate pentahydrate (0.9 mg, 0.035 mmol, 0.1 eq.) in water (0.14 mL) and methanol (0.07 mL). The reaction mixture was stirred vigorously at 25°C for 1 hour. When the starting material was consumed, the reaction mixture

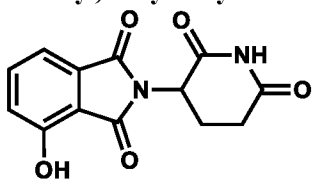
was diluted with 2 mL methylene chloride and washed with diluted sodium chloride solution two times. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The crude product was purified by preparative RP-HPLC using a gradient method on a Gemini® 5µm C18 110 Å column (H_2O:MeCN = 95:5 (0.1 % HCOOH) to 100% MeCN (0.1 % HCOOH)). Compound **RU231** was isolated as a yellow solid (14.6 mg, 35 %).

LC-MS ESI $[M+H^+]^+$ = 1192;

^1H NMR (600 MHz, $CDCl_3$): δ 10.84 – 10.81 (m, 1H), 9.74 – 9.63 (m, 1H), 9.23 (s, 1H), 8.70 (s, 1H), 8.48 (d, J = 5.2 Hz, 1H), 8.46 (d, J = 3.7 Hz, 1H), 8.34 (dt, J = 8.0, 1.9 Hz, 1H), 8.23 (s, 1H), 8.25 – 8.20 (m, 1H), 7.94 (d, J = 8.7 Hz, 1H), 7.82 (s, 1H), 7.73 – 7.72 (m, 1H), 7.70 (dd, J = 7.9, 1.8 Hz, 1H), 7.67 (ddd, J = 8.3, 7.3, 1.0 Hz, 1H), 7.65 – 7.63 (m, 1H), 7.62 – 7.56 (m, 1H), 7.51 (dd, J = 8.2, 3.1 Hz, 1H), 7.46 (d, J = 7.3 Hz, 1H), 7.46 – 7.40 (m, 2H), 7.14 (d, J = 5.1 Hz, 1H), 7.12 (d, J = 8.5 Hz, 1H), 7.11 (s, 1H), 5.34 – 5.25 (m, 2H), 5.02 – 4.94 (m, 1H), 4.63 – 4.54 (m, 2H), 4.51 (t, J = 5.1 Hz, 2H), 3.83 (t, J = 5.0 Hz, 2H), 3.66 – 3.53 (m, 14H), 3.53 – 3.49 (m, 2H), 2.87 – 2.81 (m, 1H), 2.80 – 2.73 (m, 2H), 2.71 – 2.57 (m, 2H), 2.53 – 2.46 (m, 1H), 2.33 (s, 3H), 2.15 – 2.09 (m, 1H), 2.05 – 1.97 (m, 1H), 1.52 (s, 3H).

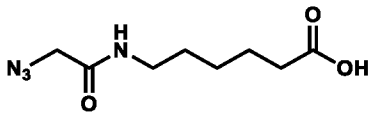
^{13}C NMR (151 MHz, $CDCl_3$): δ 199.6, 199.5, 172.7, 171.62, 171.59, 168.71, 168.70, 166.9, 166.8, 166.68, 166.67, 165.8, 165.2, 162.6, 161.79, 161.77, 160.9, 160.64, 160.62, 159.0, 154.3, 151.4, 148.4, 141.9, 138.00, 137.99, 136.9, 136.00, 135.99, 134.6, 134.3, 133.65, 133.57, 132.7, 130.3, 130.2, 129.44, 129.43, 129.02, 129.01, 125.1, 124.95, 123.94, 123.7, 123.5, 123.97, 122.95, 122.57, 122.55, 119.3, 118.7, 118.61, 118.59, 117.9, 117.2, 107.9, 70.55 – 70.22 (m), 69.4, 69.2, 67.8, 67.8, 59.0, 50.3, 49.3, 45.1, 39.0, 35.7, 31.9, 31.4, 24.3, 22.7, 18.3.

2-(2,6-dioxopiperidin-3-yl)-4-hydroxyisoindoline-1,3-dione (53)



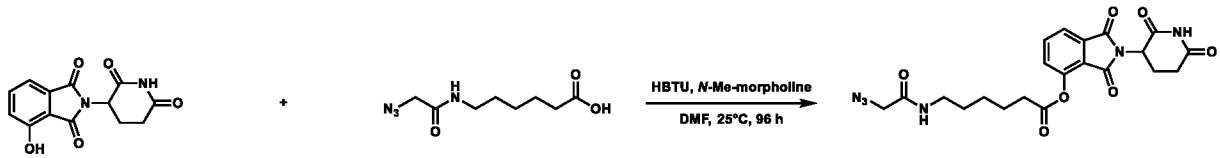
Compound **53** was synthesized according to literature procedure (Robb et al., 2017).

6-(2-azidoacetamido)hexanoic acid (54)



Compound **54** was synthesized according to literature procedure from 6-aminohexanoic acid (Ha et al., 2017).

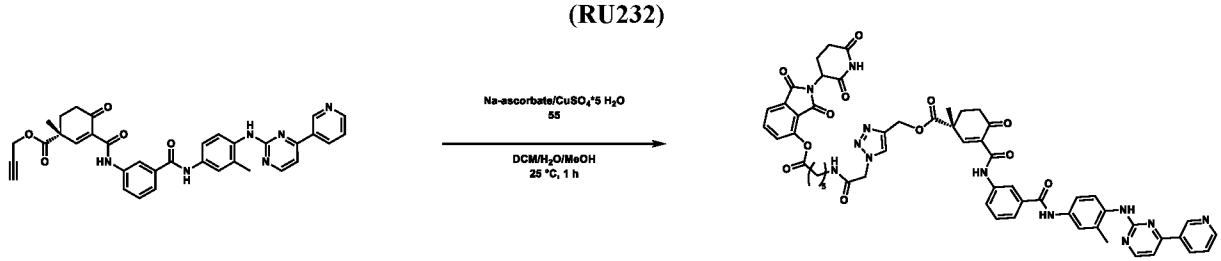
Preparation of 2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl 6-(2-azidoacetamido)hexanoate (55)



Compound **54** (69.8 mg, 0.274 mmol, 1.5 eq.) was dissolved in anhydrous dimethylformamide (0.5 mL) in a flame-dried vial under a nitrogen atmosphere, HBTU (103.7 mg, 0.274 mmol, 1.5 eq.) and *N*-methylmorpholine (60 µL, 0.547 mmol, 3.0 eq.) were added and the mixture was stirred at 25°C. After 30 minutes compound **53** (50.0 mg, 0.182 mmol, 1 eq.) was added and the reaction mixture was stirred at 25°C for 4 days. The reaction was monitored by HPLC-MS and when the starting material was consumed the reaction mixture was diluted with ethyl acetate, washed two times with a 5% citric acid solution, two times with a saturated aqueous Na_2CO_3 solution, and two times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure The residue was purified by flash column chromatography on silica gel using methylene chloride: methanol = 99:1 as eluent. Compound **55** was obtained as a yellow solid (55.8 mg, 65.1%).

LC-MS ESI $[M+H^+]^+$ = 471;

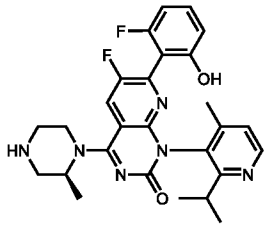
Preparation of (1-(2-((6-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)-6-oxohexyl)amino)-2-oxoethyl)-1*H*-1,2,3-triazol-4-yl)methyl (1*R*)-1-methyl-3-((3-((3-methyl-4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)carbamoyl)phenyl)carbamoyl)-4-oxocyclohex-2-ene-1-carboxylate: (RU232)



To a solution of compound **RU144** (23.5 mg, 0.038 mmol, 1.5 eq.) and compound **55** (12.0 mg, 0.025 mmol, 1 eq.) in methylene chloride (0.10 mL) was added a solution of sodium-ascorbate (2.5 mg, 0.012 mmol, 0.5 eq.) and copper(II) sulfate pentahydrate (0.6 mg, 0.0025 mmol, 0.1 eq.) in water (0.10 mL) and methanol (0.05 mL). The reaction mixture was stirred vigorously at 25°C for 1 hour. When the starting material was consumed, the reaction mixture was diluted with 1 mL methylene chloride and washed with diluted sodium chloride solution two times. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The residue was purified by reverse phase column chromatography on modified C18 using acetonitrile - water eluent. Compound **RU232** was isolated as a yellow solid (6.0 mg, 20 %).

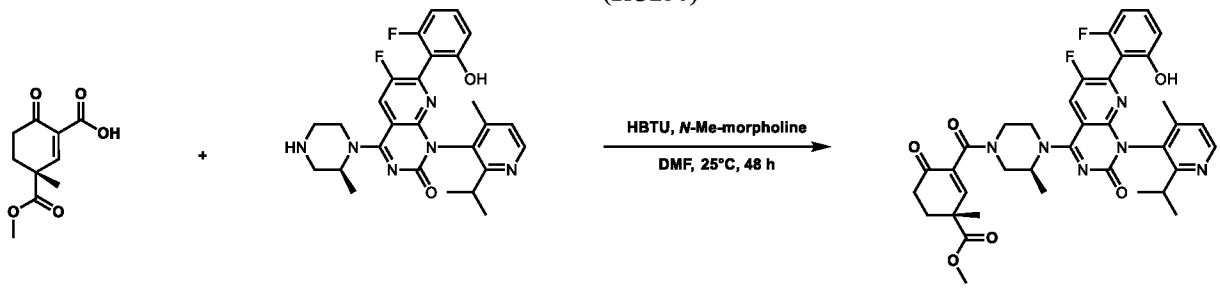
LC-MS ESI $[M+H^+]^+$ = 1086;

6-fluoro-7-(2-fluoro-6-hydroxyphenyl)-1-(2-isopropyl-4-methylpyridin-3-yl)-4-((*S*)-2-methylpiperazin-1-yl)pyrido[2,3-d]pyrimidin-2(1*H*)-one (56)



Compound **56** was purchased from Advanced ChemBlocks Inc, USA.

Preparation of methyl (1*R*)-3-((3*S*)-4-(6-fluoro-7-(2-fluoro-6-hydroxyphenyl)-1-(2-isopropyl-4-methylpyridin-3-yl)-2-oxo-1,2-dihydropyrido[2,3-d]pyrimidin-4-yl)-3-methylpiperazine-1-carbonyl)-1-methyl-4-oxocyclohex-2-ene-1-carboxylate: (RU230)



Compound ***R*-RU57** (10.1 mg, 0.047 mmol, 1.2 eq.) was dissolved in anhydrous dimethylformamide (0.08 mL) in a flame-dried vial under a nitrogen atmosphere, HBTU (18.0 mg, 0.047 mmol, 1.2 eq.) and *N*-methylmorpholine (13 µL, 0.825 mmol, 3.0 eq.) were added and the mixture was stirred at 25°C. After 30 minutes an anhydrous DMF solution (0.08 mL) of compound **56** (20.0 mg, 0.039 mmol, 1 eq.) was added and the reaction mixture was stirred at 25°C for 24 hours. After 24 hours a solution of ***R*-RU57** (10.1 mg, 0.047 mmol, 1.2 eq.), HBTU (18.0 mg, 0.047 mmol, 1.2 eq.) and *N*-methylmorpholine (13 µL, 0.825 mmol, 3.0 eq.) in anhydrous dimethylformamide under a nitrogen atmosphere was added after being stirred for 30 minutes at 25°C (additional preformed active ester). The reaction was monitored by HPLC-MS and when the starting material was consumed the reaction mixture was diluted with ethyl acetate, washed two times with a 5% citric acid solution, two times with a saturated aqueous Na_2CO_3 solution, and two times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, then evaporated under reduced pressure. The resulting crude product was purified by preparative RP-HPLC using a gradient method on a Gemini® 5µm C18 110 Å column (H_2O:MeCN = 95:5 (0.1 % HCOOH) to 100% MeCN (0.1 % HCOOH)). Compound **RU230** was obtained as a yellow solid (6.6 mg, 24 %).

LC-MS ESI $[M+H^+]^+$ = 701;

Example 2: Assays of the Compounds

Methods

Proteins, peptides, and inhibitors
Activated ERK2, p38α, and JNK1 for the biochemical assays were produced by co-expressing the MAPKs with constitutively active GST-tagged MAP2Ks in *E. coli* with bicistronic plasmids (GST-MKK1_3D/ERK2, GST-MKK6_EE/p38α, GST-MKK7_EE/JNK1/2). Double phosphorylation of the activation loop of MAPKs was confirmed by western blot analysis with anti-phospho MAPK-specific antibody and/or mass spectrometry. MAPKs were expressed with an N-terminal His6-tag that was cleaved off by the TEV protease after purification with Ni-NTA affinity resin, and then samples were further purified using ion-exchange chromatography (on MonoQ column). Dephosphorylated MAPKs were produced with GST-tagged λ-phage phosphatase and purified similarly. Human JNK3 was expressed alone using a simpler bacterial expression vector, and its activated form was produced by incubating the protein with constitutively active version of MKK7 in vitro before kinase activity measurements. GST-MKK7EE-His_6 was expressed in bacteria and double-affinity purified. Biotinylated MAPKs for the SPR experiments were co-expressed with the BirA ligase in *E. coli* and purified as described above but this construct retained an N-terminal AviTag after TEV cleavage. Peptides were chemically synthesized using solid phase peptide synthesis (on Rink Amid resin, PS3 peptide synthesizer, Protein Technologies) with Fmoc/^{t}Bu strategy and purified by RP-HPLC using a Jupiter 300 Å C18 column (Phenomenex). Quality of the peptides were checked by HPLC-MS (Shimadzu LCMS-2020) JNK-IN-8 was purchased from Selleck Chemicals, USA. *Tert*-Butyl acrylate was purchased from Sigma-Aldrich Chemicals, USA

Protein-peptide binding assay
Fluorescence polarization assay (FP) based protein-peptide binding experiments were used to assess binding into the MAPK D-groove (Garai et al., 2012). Known MAPK docking groove binding peptides were N-terminally labeled by carboxyfluorescein (EvJIP1: PPRRPKRPTSLDLPSTPSL for JNK1 and RHDF1: SLQRKKPPWLKLDIPS for ERK2 and p38α) and 50-100 nM labeled reporter peptide was mixed with the MAPK in a concentration to achieve ~50-80% complex formation. The unlabeled competitor (peptide or small molecule) was added in increasing amounts and the FP signal was measured in a Cytation 3 (BioTek Instruments) fluorescence plate reader in 384-well plates (CORNING, Low Volume, Round Bottom, Non-Binding Surface) in 20 µL volume. The binding buffer contained the following: 20 mM Tris pH=8.0, 100 mM NaCl, 0.05 % Brij35. The K_i for each competitor was determined by fitting the data to a competition binding equation. Titration experiments were carried out in triplicates, and the average FP signal was used for fitting the data in Origin 2018 (OriginLab, USA). The standard weighted least squares method was used to determine the K_i estimate, with its error given by the corresponding diagonal element of the covariance matrix.

In vitro kinase assay (PhALC) and in vitro IC_{50} determination
The Phosphorylation-Assisted Luciferase Complementation assay (PhALC) was developed to measure MAPK activity *in vitro* for fast and cost-effective identification/characterization of any compound blocking MAPK activity (Poti et al, 2023). The principle of this assay is the following: the general MAPK phosphorylation target motif (S/TP) is positioned C-terminal from a MAPK binding D-motif and this SENSOR construct is fused with the small fragment of the luciferase enzyme. In the Recognition Construct (RC), the WW domain (Pin1 protein) binding specifically to the phosphorylated MAPK target motif is fused with the large fragment of the luciferase enzyme (Dixon et al., 2016) (Lu et al., 1999). Upon SENSOR phosphorylation the RC binds to the SENSOR and triggers the assembly of the luciferase enzyme which will produce photons as it turns over its substrate (coelenterazine). Both constructs are produced with an N-terminal maltose binding protein (MBP) and a C-terminal histidine-tag for high-yield bacterial expression and for simple affinity-resin purification. The two purified constructs (SENSOR and RC) were mixed with activated MAPKs, the reaction was started by injecting ATP into the reaction mix containing the luciferase enzyme substrate coelenterazine, and the luminescence signal was monitored in time. The PhALC assay was used as a semi-high throughput, microplate compatible biochemical assay to obtain IC_{50} values of any compounds blocking MAPK activity. RC and SENSOR were typically used in 1 µM concentrations with 1-10 nM double-phosphorylated MAPKs produced and purified as described earlier (Zeke et al., 2015). The coelenterazine concentration was 200 µM and the reaction was started by adding 0.1-1 mM ATP. The kinase assay buffer contained 50 mM HEPES pH=7.4, 150 mM NaCl, 0.1 % IGEPAL, 5 mM MgCl2, and 0 mM or 10 mM GSH. The luminescence signal was monitored up to 30 minutes and the slope at the linear range (typically up to 5 minutes) was calculated based on linear regression. The p38 and ERK D-SENSOR contained the CNK3 (LKKEKSAILDLYIPP) or MEF2A D-motif (SRKPDLRVVIPP) and the JNK D-SENSOR had the more JNK-specific pepPDE4B motif (GDGISRPTTLPLTTLP) (Garai et al., 2012) (Zeke et al., 2015). SENSORs contained the same MAPK phosphorylation target sequence compatible with WW domain binding: VPRTPVS. This sequence motif was positioned C-terminal from the D-motif in D-SENSORs, separated by a flexible linker (HMGSGSSGGSSGSGSVD). For IC_{50} determination the competitor was added in increasing concentrations and the normalized slope of the luminescence signal was fitted to the Dose-response equation in Origin 2018 (OriginLab, USA). All IC_{50} values were calculated based on three independent measurements (N=3) where the mean was used for

the analysis. The standard weighted least squares method was used to determine the IC_{50} estimate, with its error given by the corresponding diagonal element of the covariance matrix.

Protein-small molecule binding experiments with SPR

For surface plasmon resonance (SPR) measurements ERK2 was captured on a Biacore CM5 sensor chip by amine coupling using a Biacore S200 instrument (GE-Healthcare). The SPR running buffer was the following: 1 X PBS supplemented with 0.05% Tween20, 0.2 mM TCEP, 5% DMSO. For p38α measurements, the protein was expressed with an N-terminal His_{10}-tag and an NTA sensor chip was used for capturing the protein, and the SPR running buffer was the following: 10 mM Tris pH 8.0, 300 mM NaCl, 0.2 mM TCEP, 0.05% Tween20. For JNK1 measurements the proteins were expressed biotinylated with an N-terminal AviTag, expressed in bacteria, purified, and immobilized on a Biacore CAP sensor chip, and the SPR running buffer was the following: 10 mM HEPES pH 7.4, 150 mN NaCl, 0.05% Tween20, 1 mM GSH, 1% DMSO. All measurements were done by using a Biacore S200 instrument (GE-Healthcare) at room temperature. The K_D was determined based on RU values corresponding to different concentrations of the analyte using single-cycle setup with the standard Biacore method for CAP chip including double referencing. Sensorgrams were fit to a 1:1 binding model using the BiaEvaluation software (GE Healthcare). The kinetic binding parameters (k_{off}) were determined from sensorgrams obtained at an analyte concentration corresponding to the equilibrium binding constant (K_D). The SPR running buffer was the following: 1 mM HEPES, 150 mM NaCl, 0.05 % TWEEN, 1 mM GSH, 1% DMSO. For JNK1 measurements the protein was expressed biotinylated on an N-terminal AviTag, expressed in bacteria, purified, and immobilized on a Biacore CAP sensor chip. The determination of k1, k2 (noncovalent k_{on} and k_{off}, respectively) and k3, k4 (covalent k_{on} and k_{off}, respectively) were determined the following way. Inhibitors were injected over the JNK1(Cys116Ser) mutant surface at three different concentrations (100 nM, 300 nM, and 1000 nM) and this kinetic data was globally fit with to 1:1 binding model using the BiaEvaluation software (GE Healthcare), which gave the k1 and k2 value for each inhibitor. Next, the inhibitors were injected at three different concentrations (depending on their estimated K_D determined earlier by independent equilibrium measurements) over the CAP sensor chip loaded with wild-type JNK1 and k3 and k4 values were determined by fitting the kinetic binding curves using the COPASI biochemical modeling software based on a 2-step reversible binding scheme, where k1 and k2 values were fixed (Hoops et al, 2006).

Mass spectrometry analysis

The molecular weights of MAPKs and their conjugates were identified using a Triple TOF 5600+ hybrid Quadrupole-TOF LC/MS/MS system (Sciex, Singapore, Woodlands) equipped with a DuoSpray IonSource coupled with a Perkin Elmer Series 200 micro LC system (Massachusetts, USA). Data acquisition and processing were performed using Analyst TF software version 1.7.1 (AB Sciex Instruments, CA, USA). Chromatographic separation was achieved by Thermo Beta Basic C8 (50 mm × 2,1mm, 3 µm, 150 Å) HPLC column. Sample was eluted in gradient elution mode using solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in ACN).

The molecular weights of the conjugates of GSH were identified using a Triple TOF 5600+ hybrid Quadrupole-TOF LC/MS/MS system (Sciex, Singapore, Woodlands) equipped with a DuoSpray IonSource coupled with a Shimadzu Prominence LC20 UFLC (Shimadzu, Japan) system consisting of binary pump, an autosampler and a thermostated column compartment. Data acquisition and processing were performed using Analyst TF software version 1.7.1 (AB Sciex Instruments, CA, USA). Chromatographic separation was achieved on a Phenomenex Luna Omega PS C18 (50 mm × 2,1mm, 3 µm, 100 Å) HPLC column. Sample was eluted in gradient elution mode using solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in ACN). The initial condition was 10% B followed by a linear gradient to 55 % B by 12 min, to 95 % B by 3 min, 15 to 17.5 min 95% B was retained; and from 17.5 to 18 min back to initial condition with 10 % eluent B and retained from 18 to 20 min. Flow rate was set to 0.4 ml/min. The column temperature was 40 °C and the injection volume was 5 µl. UV-VIS spectrometer was used in 254 nm wavelength. Nitrogen was used as the nebulizer gas (GS1), heater gas (GS2), and curtain gas with the optimum values set at 30, 30 and 35 (arbitrary units), respectively. Data were acquired in positive electrospray mode in the mass range of m/z=300 to 2500, with 1 s accumulation time. The source temperature was 350 °C and the spray voltage was set to 5500 V. Declustering potential value was set to 80 V. Peak View Software™ V.2.2 (version 2.2, Sciex, Redwood City, CA, USA) was used for deconvoluting the raw electrospray data to obtain the neutral molecular masses.

NanoBRET target engagement assay

The NanoBRET target engagement assay was carried out by Reaction Biology Corp (USA, Malvern). Briefly, HEK293 cells were transfected with 1 µg JNK1 and 9 µg transfection carrier DNA. The transfected cells were treated with compounds (starting at 1 or 100 µM, 10-dose with 3-fold dilution). JNK1 target engagement was measured by NanoBRET assay. The K-5 tracer concentration was 0.5 µM. Compound treatment time was 2 hours.

NanoBiT protein-protein interaction assay

For luciferase complementation NanoBiT assays (Promega), HEK293T cells were transfected with LgBiT and SmBiT containing plasmids using Lipofectamin 3000 in Opti-MEM (Gibco). cDNAs were sub-cloned into LgBiT and SmBiT expression vectors: ERK2 and p38α were expressed as LgBiT fusions and partner proteins constructs had Smbit fusion tags. Cells were serum-starved for overnight in DMEM (Gibco) and luciferase activity was measured in 96-well white

plates in a luminescence plate reader (Cytation 3, BioTek) at 37 °C after the addition of 20 µM Coelenterazine h (301-10 hCTZ; Prolumen Ltd, USA) at the maximum level of luminescence (3-8 minutes).

Cell culture

25,000 HeLa cells were seeded into 48-well plates and were grown till 80 % confluence (typically 24 hrs) in 200 µl DMEM supplemented with 10 % FBS and were serum-starved (0 % FBS) for 16 hours before pretreatment with inhibitors (10 µM) for 1.5 hour. Cells were stimulated by 100 ng/mL EGF for the indicated time, the media was removed, and cells were lysed in 70 µl 1X SDS-PAGE loading buffer, 10 µl was loaded onto 4-20 % Mini-PROTEAN TGX Precast gels (Bio-Rad) 5-15 % Tris-glycine SDS-PAGE gels and gels were blotted to nitrocellulose membrane. The DMSO control contained 0.05 % DMSO, an equal amount to inhibitor treated cells. Western blot results were analyzed using Odyssey CLx imaging system (Li-Cor) and fluorescently labeled secondary antibodies: IRDye 680RD (Goat anti-mouse IgG; Li-cor #925-68070; 1:10000) or IRDye 800CW (Goat anti-rabbit IgG, Li-Cor #926-32211; 1:5000). The primary antibodies were the following: anti-p44/42 MAPK (ERK1/2) (L34F12) Mouse mAb (Cell Signaling #4696; 1:3000 dilution; referred to as ERK antibody), anti-phospho-p44/42 MAPK (Thr202/Tyr204) Rabbit Ab (Cell Signaling #9101; 1:3000 dilution; referred to as ppERK), anti-phospho-p90RSK (S380) (D3H11) Rabbit mAb (Cell Signaling 11989; 1:2000 dilution; referred to as pRSK), anti-α-tubulin (Sigma #T6199; 1:10000 dilution, referred to as TUB).

50,000 HEK293T cells were seeded into 24-well plates and were grown till confluence (typically 24 hrs) in 500 µl DMEM supplemented with 10 % FBS and were serum-starved (0% FBS) for 16 hours before sorbitol (0.35 M) treatment for 15 minutes. The plate was put on ice and cells were resuspended, pelleted by centrifugation, and washed with ice cold PBS. The cell pellet was lysed in 80 µl 1X SDS-PAGE loading buffer, 10 µl was loaded onto 10 % Tris-glycine SDS-PAGE gels and gels were blotted to nitrocellulose membrane. Inhibitors (1 µl dissolved in 50 % DMSO) were added 2 hours before sorbitol treatment. SH-SY5Y MKK7 ACT cells, allowing specific activation of JNK upon addition of an inducer, were handled similarly but JNK activation was induced by the addition of 3 µg/ml doxycycline. Inhibitors (1 µl dissolved in 50 % DMSO) were added together with doxycycline and samples were harvested for western blot analysis as described above. Western blot results were analyzed using Odyssey CLx imaging system (Li-Cor) and fluorescently labeled secondary antibodies: IRDye 680RD (Goat anti-mouse IgG; Li-cor #926-68070; 1:10000) or IRDye 800CW (Goat anti-rabbit IgG, Li-Cor #926-32211; 1:5000). The primary antibodies were the following: anti-phospho JNK (Cell Signaling #9251; 1:1000), anti-phospho-c-Jun(Ser73) (Cell Signaling #9164; 1:1000), anti-phospho p38 (Cell Signaling #9215, 1:3000), anti-phospho MK2 (Cell Signaling #3007, 1:1000), anti-α-tubulin (Sigma #T6199; 1:10000), or anti-FLAG (Sigma #F1804; 1:10000). Anti-α-tubulin antibody was used as the load control and the anti-FLAG antibody was used to monitor the expression of the transgene responsible for specific MAPK activation.

AP-1 Reporter HEK293 cells (BPS Bioscience) contains a firefly luciferase gene under the control of AP-1 responsive elements that are stably integrated into HEK293 cells. For AP-1 promoter mediated transcription measurements, 20,000 Reporter AP-1 – HEK293 cells were seeded into a 48-well plate in 200 µl DMEM (Gibco). After cells become adherent (confluency: 80 %) the inhibitors were added in opti-MEM (Gibco) and following 2 hours of incubation cells were stimulated with 6 ng/ml PMA solution for 6 hours. The luminescence signal was read out using a BioTek Cytation 3 microplate reader after adding freshly prepared Steadylite Plus solution (PerkinElmer) according to the manufacturer's protocol.

The SH-SY5Y (neuroblastoma) MKK7 ACT cell line was generated using lentiviral transduction with an MKK7(ACT)-MLK3 construct containing full-length human MKK7 fused to the kinase domain of human MLK3 (aa. 117-379). The generated Tet-ON inducible stable cell line was evaluated by time dependent transgene induction and downstream target phosphorylation upon doxycycline treatment, as described earlier (Kirsch et al., 2020). For the determination of JNK inhibitor mediated c-Jun phosphorylation, cells were stimulated with 2 µg/mL doxycycline, co-administered with different amounts of inhibitors (between 30 µM and 30 or 10 nM concentrations in three-fold dilutions with 7 or 8 data points), and samples were subjected to quantitative western blot (WB) analysis after 6 hours. To obtain the p-c-Jun EC_{50} value, the nomalized phospho-c-Jun WB signal was fitted with the DoseResponse function of Origin 2018. The values for JNK-IN-8, BD837-IN-8, RU144-IN-8, and RU155-IN-8 were determined based on three indpenedent experiments (N=3), while values for other compounds in Table 9 are based on only one experiment (N=1). The standard weighted least squares method was used to determine the EC50 estimate, with its error given by the corresponding diagonal element of the covariance matrix.

The PrestoBlue Cell Viability Reagent (ThermoFisher, P50200) was used to assess cell viability of SH-SY5Y MKK7 ACT cells. 3,000 SH-SY5Y MKK7 ACT cells were plated in 96-well plates in DMEM with 10% FBS. Next day the medium was changed for new media (DMEM with 0.1 % of FBS) and cells were incubated with inhibitors for 72 hours. The medium was then removed and PrestoBlue reagent was added to live cells, incubated for 45 minutes, then the fluorescence signal (ex: 560 nm, em: 600 nm) was measured in a plate reader. Linearity of the detection was checked in a parallel experiment where control cells were serially diluted, and the signal was measured according to the manufacturer's protocol.

The PrestoBlue Cell Viability Reagent (ThermoFisher, P50200) was used to assess cell viability of A549, H1792 and LCL-103H human cancer cell lines. 1000 cells were plated in 96-well plates in DMEM with 10% FBS. Next day the medium was changed for new media (DMEM with 0.5 % of FBS) and cells were incubated with inhibitors for 72 hours. The medium was then removed and PrestoBlue reagent was added to live cells, incubated for 45 minutes, then

the fluorescence signal (ex:560 nm, em: 600 nm) was measured in a plate reader. Linearity of the detection was checked in a parallel experiment where control cells were serially diluted, and the signal was measured according to the manufacturer's protocol.

Kinase specificity profiling

The human kinase specificity profile of BD837-IN-8 was tested by Reaction Biology Europe GmbH (Freiburg, Germany) using the Wild Type Kinase Panel comprised of 340 active human kinases. Briefly, profiling was done using the ^{33}PanQinase™ assay which measures the inhibition of phosphorylation of kinase substrate peptides in a radioactive assay format (Wang and Ma, 2015). The data is given as per cent remaining activity compared to the control experiment without the inhibitor. The inhibitor was used in 1 μM concentration and ATP was used at a concentration matching its apparent K_M for each kinase. Test compound, kinase, ATP, and a natural substrate peptide for each kinase were incubated in FlashPlate microtiter plates (PerkinElmer) coated with a scintillant. Reactions were stopped after one hour. Assays were carried out in duplicates.

PROTAC experiments

100,000 HeLa cells were seeded into 24-well plate in DMEM supplemented with 10 % FBS. The next day the media was changed to DMEM. PROTACs and/or the proteosome inhibitor (MG132) were added in 10 μM concentrations and cells were incubated for 24 hours. Cells then were lyzed in 100 μL 1.5 X SDS loading buffer and samples (10 μL) were subjected to western blot analysis. Western blot results were analyzed using Odyssey CLx imaging system (Li-Cor) and fluorescently labeled secondary antibodies: IRDye 680RD (Goat anti-mouse IgG; Li-cor #926-68070; 1:10000) or IRDye 800CW (Goat anti-rabbit IgG, Li-Cor #926-32211; 1:5000). The primary antibodies were the following: anti-JNK (Cell Signaling #3708; 1:3000), anti-p38 (Cell Signaling #9212; 1:3000 and anti-α-tubulin (Sigma #T6199; 1:10000). Anti-α-tubulin antibody was used as the load control.

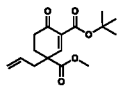
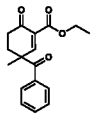
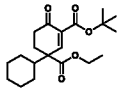
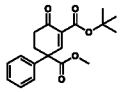
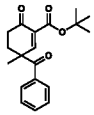
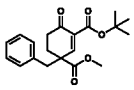
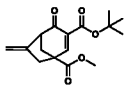
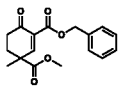
Results

Most kinase inhibitors bind in the deep nucleotide binding crevice of protein kinases and block kinase activity. These ATP-competitive drugs need to bind with high affinity (low nanomolar) as they compete against the millimolar concentration of ATP in the cell. The presence of a surface cysteine on the target around the ATP-pocket may be exploited to increase binding affinity and target residence time, and thus inhibitor potency, by linking the noncovalent ATP-pocket binding moiety to a covalent warhead (Zhao et al., 2017) (Chaikuad et al., 2018). The specificity of the composite inhibitor comes mostly from noncovalent contacts which are formed via the ATP-pocket binding moiety. Protein kinases provide an excellent test system to compare currently used and new warhead scaffolds as cysteine attacking anchors for several reasons: 1) there are several well-documented examples of acrylamide warhead containing ATP-competitive inhibitors targeting these proteins (Boike at al, 2022), 2) the great structural similarity of members from this big family of enzymes allows comparative studies on specificity mechanisms, where negative selection against off-targets may be just as important as positive selection for the target, and 3) many kinases depend on intact protein-protein interactions mediated through shallow surface (docking) grooves that are difficult to target by small molecules (Miller et al, 2018). Protein kinases could be used to assess the benefits of new chemical warheads, which in turn could be applied on less-characterized protein targets.

To identify surface cysteine targeting new Michael acceptor warheads that could be used to block mitogen-activated protein kinase (MAPK) signaling, we used a biochemical assay that monitored binding to the D(ocking)-groove of MAPKs where interference with MAPK-reporter peptide binding was indicative of compound reactivity to a surface cysteine (**FIGURE 1**) (Garai et al., 2012). We collected compounds with different Michael acceptor warheads comprised of building block like molecules which were originally used for the chemical synthesis of complex Michael acceptor group containing natural products or pharmaceutical drugs (Berkes et al., 2016) (Martin et al., 2020). In addition to the compounds used as intra-chain acrylamide warheads (Group 1), the collection contained 75 compounds representing 7 types of Michael acceptors. Each group contained ~5 to 20 different molecules where substituent groups varied: 2) cyclohexenone, 3) frustrated cyclohexenone, 4) acryl - chalcone, 5) cyanoacryl, 6) frustrated cyanoacryl and 7) nitroalkene. The reactivity of the beta carbon in the different groups varies, as the chemical environment of α,β-unsaturated carbonyls affect the electronic properties of the warhead, while in frustrated compounds (Group 3 and 6) reactivity is also affected by steric hindrance. The collection was used to search for molecules capable of interfering with the binding of fluorescently labeled D-motif peptides to ERK2, p38α or JNK1. Group 3 (frustrated cyclohexenone) and Group 7 (nitroalkene) compounds showed interference with reporter D-motif peptide binding. Group 7 compounds were found ineffective when the same screen was repeated under high amounts of GSH (10 mM), while, unexpectedly, Group 3 molecules retained their inhibitory effect. Frustrated cyclohexenone compounds, referred to as RU compounds with a specific number designation henceforth (e.g., RU1 or RU46), were further tested. Interestingly, compounds showed varying degrees of selectivity towards the three MAPKs tested (e.g., RU42: ~20-fold difference for p38 versus ERK2; RU62: >10-fold for p38 versus JNK1, and RU41: >5-fold for JNK1 versus ERK2 binding). While all compounds bound to p38α the best (~ 0.5-10 μM), some molecules - depending on the substituent groups - also bound to ERK2 and JNK1 with medium micromolar (2-10 μM) affinity (**TABLE 1**). We further characterized ERK2 binding

of frustrated cyclohexenone compounds and the contribution of different substituent groups to ERK2 binding was examined. This structure-activity relationship analysis (SAR) established the importance of the electron withdrawing ester group in C2 and C4 next to the α,β-unsaturated carbonyl (C3), and it also showed that bulky hydrophobic groups in R'2, connected to C2 via an ester, increase binding affinity (**FIGURE 2A**). Conversely, the mutation of Cys161 to alanine greatly reduced binding affinity as expected (**FIGURE 2B**).

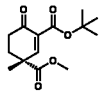
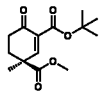
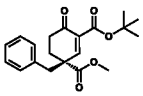
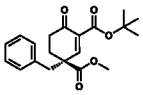
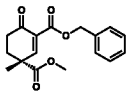
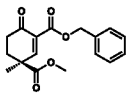
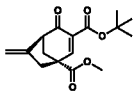
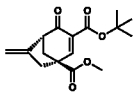
TABLE 1 shows different compounds with their MAPK binding affinity. K_i values, Ki_{app}, were determined in competitive protein–D-peptide binding assays with ERK2, p38α, and JNK1. (* Not claimed, for reference only; parameter error estimate from weighted least squares method, N=3)

		ERK2 Ki_{app}/μM	p38 Ki_{app}/μM	JNK1 Ki_{app}/μM
RU41		15.7 ± 1.0	2.7 ± 0.2	2.9 ± 0.7
RU42		172.0 ± 30.4	8.5 ± 0.7	> 200
RU43		16.0 ± 1.2	1.6 ± 0.2	7.4 ± 1.3
* RU44		> 200	> 200	> 200
RU45		144.3 ± 22.3	10.9 ± 0.5	35.3 ± 5.4
* RU56		> 200	> 200	> 200
* RU57		> 200	>200	> 200
RU61		26.5 ± 2.0	4.0 ± 0.5	47.0 ± 8.3
RU62		11.3 ± 0.5	1.2 ± 0.1	18.8 ± 3.9
RU63		10.9 ± 1.2	2.2 ± 0.2	14.5 ± 2.7
RU64		4.3 ± 0.2	0.5 ± 0.1	4.8 ± 1.1
RU70		12.7 ± 1.2	0.8 ± 0.1	2.1 ± 0.2
RU72		16.2 ± 1.0	5.4 ± 0.4	4.0 ± 0.0
* RU146		31.2 ± 1.3	19.0 ± 1.6	110.3 ± 14.5

Compounds contain an asymmetric center and we also measured MAPK binding of the different enantiomers in addition to the racemic mixtures used in former tests. Compounds were produced by stereoselective synthesis. Different stereoisomers with one chiral center bound to MAPKs with modest binding affinity difference (<5-fold), however, the

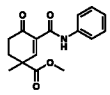
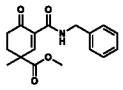
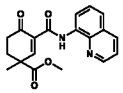
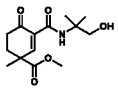
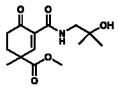
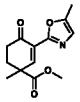
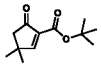
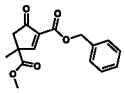
stereoisomers of a molecule (RU70) containing two chiral centers (RU73-*S,S* and RU77-*R,R*) bound with ~8-10-fold difference (**TABLE 2**).

TABLE 2 shows the MAPK binding affinity of different stereoisomers of some selected compounds. K_i values, Ki_{app}, were determined in competitive protein–D-peptide assays with ERK2, p38α, and JNK1. (Parameter error estimate from weighted least squares method, N=3)

		Configuration	ERK2 Ki_{app}/μM	p38 Ki_{app}/μM	JNK1 Ki_{app}/μM
RU60		*R*	12.3 ± 0.4	1.7 ± 0.1	23.6 ± 1.5
RU187		*S*	4.9 ± 0.2	8.3 ± 0.7	6.5 ± 0.6
RU67		*R*	6.3 ± 0.3	0.8 ± 0.1	1.8 ± 0.4
RU68		*S*	5.9 ± 0.4	0.8 ± 0.1	1.2 ± 0.3
RU75		*R*	10.1 ± 1.0	4.8 ± 0.4	27.6 ± 3.9
RU76		*S*	24.7 ± 1.5	5.5 ± 0.6	15.3 ± 2.4
RU73		*S,S*	66.7 ± 5.1	6.2 ± 0.4	8.0 ± 0.7
RU77		*R,R*	8.4 ± 0.7	0.6 ± 0.0	1.0 ± 0.1

In contrast to acrylamide mediated irreversible adduct formation, covalent adduct formation by cyclohexenone compounds is unexpectedly reversible (**FIGURE 3A-C**). The ester group next to C2 has a strong electron withdrawing effect on C3 making the unsaturated β-carbonyl atom strongly reactive towards the sulfhydryl group of the cysteine. Quantum chemical calculations suggested that the different substituent groups at C2 would have a great effect on the enthalpy change of cysteine-adduct formation. An amide group at this position has less electron withdrawing capacity and it is expected to be less reactive to cysteine. Indeed, amide analogs of some of the ester compounds (e.g., RU58 – RU43, RU66 – RU72) or other differently substituted amide compounds (RU59, RU65, RU84, or RU85) bound weaker to all MAPKs compared to ester-containing compounds in this critical position (**TABLE 3**). Amide or oxazole containing compounds also formed a reversible covalent bond with ERK2 similarly to ester-containing warheads (**FIGURE 4C**). The examples listed in TABLE 1-3 demonstrate that the nature of the electron withdrawing group at C2 and other substituent groups at other ring positions, or the size of the ring have a great impact on the capacity of cyclic scaffolds to target the MAPK docking groove cysteine.

TABLE 3 lists the MAPK binding affinity of additional examples. K_i values, Ki_{app}, were determined in competitive protein–D-peptide binding assays with ERK2, p38α, and JNK1. (* Not claimed, for reference only; parameter error estimate from weighted least squares method, N=3)

		ERK2 Ki_{app}/μM	p38 Ki_{app}/μM	JNK1 Ki_{app}/μM
RU58		> 200	74.7 ± 14.0	> 200
RU59		> 200	19.3 ± 3.1	> 200
RU66		32.8 ± 2.7	42.2 ± 2.9	97.4 ± 24.1
RU65		71.4 ± 9.2	38.9 ± 6.2	> 200
RU84		> 200	53.0 ± 11.1	> 200
RU85		> 200	55.9 ± 6.5	> 200
RU81		42.7 ± 3.6	21.2 ± 2.8	112.2 ± 32.7
* RU151		168.7 ± 8.7	27.4 ± 3.3	> 200
RU169		37.2 ± 4.0	9.4 ± 2.1	2.1 ± 0.3

To explore the structure of the MAPK-cyclohexenone warhead adduct, we co-crystallized RU67 with ERK2 using the same protein reported previously (Garai et al, 2012). We used X-ray crystallography to determine the structure of the ERK2-RU67 complex at 2.0 Å resolution (**TABLE 4**). The compound bound to Cys161 covalently and the cyclohexenone moiety with its hydrophobic substituents occupied the hydrophobic pockets (φL, φA, and φB) similarly to a natural D-peptide (**FIGURE 4**).

TABLE 4 shows the details of protein crystallization, crystallographic data collection, and structure solution for the ERK2-RU67 covalent complex.

	ERK2-RU67
Crystallization conditions[a]	30% PEG1000, 0.1M HEPES pH 7.5
Data collection	
Space group	$P2_12_12_1$
Cell dimensions	
a, *b*, *c* (Å)	41.47, 77.39, 127.22
α, *β*, *γ* (°)	90, 90, 90
Resolution range (Å)	50.0-2.0
$CC_{1/2}$[b]	0.998 (0.871)
R_{merge}[c]	0.16 (1.68)
$I/\sigma I$	10.5 (1.6)
Completeness (%)	100 (100)

Redundancy	11.8 (11.4)
No. reflections	28489
Refinement	
R_{work} / R_{free}	0.1829 / 0.2160
No. atoms	2912
Protein	2755
Ligand/ion	52
Water	105
B-factors (Å^2)	51.74
Protein	51.85
Ligand/ion	53.62
Water	48.03
Ramachandran	
Favored (%)	96.11 %
Allowed (%)	3.89 %
Outliers (%)	0.00 %
R.m.s deviations	
Bond lengths (Å)	0.008
Bond angles (°)	0.859

[a] Crystals were grown with the vapor diffusion method and were frozen in liquid nitrogen in mother liquor supplemented with 25 % glycerol.
[b] Values in parenthesis are for the highest resolution bin.
[c] $R_{merge} = \Sigma_{hkl}\Sigma_i |I_i(hkl)-<I(hkl)>|/\Sigma_{hkl}\Sigma_i I_i(hkl)$

In summary, screening of Michael acceptor group containing compounds identified a multi-functionalized cyclohexenone scaffold as a putative covalent warhead specifically targeting the MAPK docking groove cysteine. Structure-activity relationship (SAR) analysis showed that electron withdrawing groups (e.g., ester) as substituents both at C2 and C4 - the two carbon atoms next to the unsaturated β carbon (C3; see Figure 2A) - of the cyclohexenone ring together with a hydrophobic directing moiety linked to C2 were necessary for low micromolar binding in the MAPK docking groove. Surprisingly, such a multi-functionalized cyclohexenone scaffold has the right amount of reactivity towards the MAPK docking groove cysteine for reversible covalent bond formation but a hydrophobic substituent group (e.g., *tert*-butyl or benzyl) was also needed to direct the electrophile into the hydrophobic pocket next to the docking groove cysteine.

We posit that the new cyclic warheads could be used in composite inhibitors in which the substituent groups may be extended to make additional noncovalent contacts to endow these more complex molecules (comprised of one of the new cyclic warhead scaffold designs and a directing group) with new properties, for example to increase binding towards selected cysteines and/or to achieve desirable cellular effects. Since the size of the molecules shown in Table 1-3 are small and thus could be used as alternatives to open-chain (acyclic) acrylamide- or acrylester based warheads, we tested one of the new cyclic warhead designs for its capacity to target two distinct surface cysteines – JNK1(Cys116) located in an "open" position next to the substrate binding pocket at the rim of the ATP-binding pocket and ERK2(Cys161) from the MAPK D-groove – as a cyclohexenone-amide or -ester containing composite molecule (**FIGURE 5A**). Acrylamide/ester and cyanoacrylamide/ester were used as acyclic irreversible or electronically tuned (reversible) acyclic Michael acceptors, respectively, and the warheads' performance on targeting these two distinct cysteines were compared (**FIGURE 5B**). The known JNK-specific IN-8 inhibitor scaffold was used to recruit the warheads to JNK1(C116) (Zhang et al, 2012) and the *tert*-butyl group was used as the directing moiety for ERK2(C161). This comparative analysis showed that the new cyclic warhead is comparable to, or even supersedes, its tested alternatives (irreversible acrylamide or reversible cyanoacrylamide) in the case of Cys116 in JNK1. Moreover, for Cys161 on ERK2, the new cyclic warhead performed a lot better compared to its alternatives. The stereochemical requirements for covalent bond formation as well as for noncovalent contact compatibility is far stricter and more specific around a Michael acceptor if it is part of a ring compared to when it is in an open-chain "context". Conversely, a more rigid, cyclic, and thus structurally more constrained reversible warhead may contribute more to binding affinity on its own right, far beyond that of a flexible, open-chain reversible covalent anchor (e.g. cyanoacrylamide). The advantage is more apparent when the cyclic warhead can target a cysteine in a surface pocket that matches up to its size and/or stereochemical configuration well. This positive selection mechanism is possibly the reason for the unexpectedly high efficiency of cyclohexenone-based warheads in targeting the MAPK D-groove cysteine. Conversely, the more complex ring structure can also hinder reactivity towards off-target cysteines due to higher probability for incompatibility with local surface topography (negative selection).

The crystal structure of the ERK2-RU67 complex showed that the cyclohexenone moiety fills up the φA and φB pockets, but the charged CD groove remains unoccupied (**FIGURE 6A**). This provided a great opportunity to extend the hit molecules. To produce compounds that fill this polar pocket, we extended one of the stereoisomers of RU43 using a click chemistry-based (Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC)) approach. The carboxyl group at

C4 was extended by a propargyl group (RU83), which was then clicked to peptides through an N-terminal azide (**FIGURE 6B**). In silico docking showed that the triazole group may direct the new moieties towards the CD groove by fitting into the φ_L. Indeed, when the CD-groove binding half of the natural MNK1 peptide was clicked to RU83, the binding affinity greatly increased (p38α; Ki_{app} ~ 3 nM), and this effect was MAPK-specific (**FIGURE 6C**). The increased binding affinity of the RU83-peptide chimera was due to increased residence time (decrease in k_{off}) (**FIGURE 6D**). These findings demonstrated that compounds with the reversible cyclic warhead could be rationally extended to increase MAPK binding affinity and specificity.

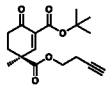
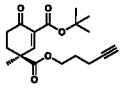
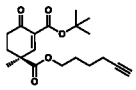
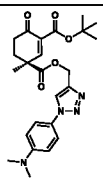
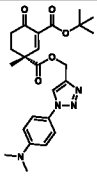
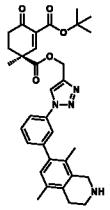
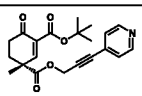
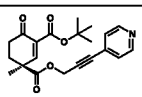
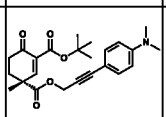
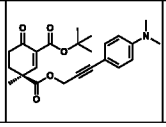
The nucleotide binding pocket of MAPKs is located close to the hydrophobic pockets of the D-groove (**FIGURE 7A**). It was shown earlier that connecting a D-peptide with an ATP pocket binding moiety can give a high-affinity bivalent ERK inhibitor (Lechtenberg et al., 2017). The enantiomer of RU83 (referred to as RU188) was linked to the ATP-binding moiety of an ATP competitive inhibitor, JNK-IN-8 (Zhang et al., 2012). While IN-8 – the ATP binding moiety of the composite JNK-IN-8 inhibitor – bound to JNK1 with only medium affinity (K_D = 228 nM), RU128 – containing IN-8 connected to RU188 with a suitable linker – bound close to 100-fold stronger (K_D = 2.4 nM) (**FIGURE 7B**). Moreover, the residence time of the bivalent compound greatly increased (~ 200-fold; based on apparent koff measurements by SPR in the presence of 1mM GSH) (**FIGURE 7C**).

We used a new in vitro kinase assay to test the effect of new compounds on MAPK docking motif directed substrate phosphorylation. The NanoBiT luciferase complementation protein-protein interaction assay concept was modified so that kinase docking facilitated phosphorylation of an artificial substrate sensor (D-sensor for MAPKs) could be qualitatively monitored in a microplate reader (Dixon et al., 2016). This Phosphorylation-Assisted Luciferase Complementation assay (PhALC) was developed as a semi high-throughput, microplate compatible biochemical assay to obtain IC_{50} values of newly developed compounds on docking-based substrate phosphorylation. Furthermore, the assay allows fast and cost-effective identification/characterization of any compound blocking MAPK activity (Poti et al, 2023). RU83-click-pepMNK1_C contains the RU83 warhead extended towards the CD-groove. Based on the crystallographic models, the configuration of the substituent groups at C4 in this small molecule is better suited for extending the propargyl group towards the CD-groove (**FIGURE 8A**). This compound was linked with the CD-groove binding region of a natural docking peptide (pepMNK1) and this RU83-peptide chimera indeed efficiently blocked D-groove dependent phosphorylation in the presence of 10 mM GSH (where the lowest IC_{50} value, 0.4 μM, was found for p38α, probably because this MAPK has the highest degree of structural plasticity at its docking groove). MAPKs may also be blocked by inhibitors that target the ATP-binding pocket. We used a MAPK ATP-pocket binding moiety from a known ATP-competitive JNK inhibitor (IN-8; Zhang et al, 2012) and linked it to the enantiomer of RU83 (RU188). Based on the structural models, the configuration at C4 in RU188 is better suited for positioning IN-8 towards the ATP-pocket. RU128, a composite inhibitor containing RU188 linked to IN-8 by using a suitable PEG linker, indeed blocked p38- or JNK-mediated activity. The IC_{50} for JNK1 was sub-micromolar (0.7 μM) even in the presence of 10 mM GSH (**FIGURE 8B**).

Next, we synthesized RU83- and RU188-based extended molecules and tested their effect on MAPK-partner protein binding in live cells. The propargyl group of these two stereoisomers were clicked to *N,N*-dimethylaniline (RU103 and RU189, respectively). The compounds were added in 10 μM concentration to HEK293T cells and their impact on the D-motif dependent MKK1-ERK2, ERK2-RSK1, and ERK2-MKP3 binding was analyzed by using a luciferase fragment complementation-based protein-protein interaction assay in live cells (NanoBiT) (Dixon et al., 2016) (Garai et al, 2012) (**FIGURE 9A**). Other experiments in HeLa cells showed that RU103 efficiently blocks EGF-triggered ERK and RSK phosphorylation, while RU189 was less efficient (**FIGURE 9B**). In further experiments MAPK-mediated transcription from the AP-1 promoter was monitored by using the AP-1 Reporter - HEK293 cell line containing an AP-1 promoter+luciferase gene cassette stably integrated into the genome (Whitmarsh et al, 1996). PMA-induced activation of AP-1 promoter mediated transcription was abrogated to a different extent depending on the nature of the extension (dimethylaniline or pyridine), its configuration, and the chemical link (1,4-substituated 1,2,3-triazole: nonlinear or arylalkyne: linear) (**FIGURE 9C**). Results with the C4-extended derivatives of the cyclohexenone core demonstrated that MAPK-binding potency depends on the configuration of the extended substituent group at the C4 stereogenic center. Unexpectedly, these findings also show that compounds perform differently on distinct D-groove dependent binary binding events due to differences in the capacity of the stereoisomers to contact additional surface residues and/or to interfere with contacts required for partner protein binding. Differential impact for example on activator kinase binding (e.g., MKK1), a deactivating phosphatase (e.g., MKP3), or specific substrates (e.g., RSK) will likely have different impact on MAPK controlled signaling events in the cell. The ester group at C4 could be extended by using azide-alkyne cycloaddition or Sonogashira coupling to add new groups directly to the cysteine targeting warhead moiety. First, this group was modified with propargyl alcohol (RU83 and RU188), which were then further extended by cycloaddition (RU103/RU189, RU115) or by Sonogashira coupling (RU140/RU141, RU214/215) (**TABLE 5**). For the MAPK D-groove cysteine, some of these extensions led to molecules that had comparable in vitro binding affinity to that of the simpler designs (see Table 3), but surprisingly they behaved as complex MAPK pathway modulators in the cell. The effect of these molecules on MAPK-based signaling networks in the cell is not straightforward to predict because they will compete with dozens, possibly hundreds of MAPK D-groove mediated interactions including binding to activator kinases, substrates, scaffolds, and/or phosphatases (i.e. partner proteins), where these latter naturally also

compete with each other and their affinity vary from submicromolar (~0.1 μM) to medium micromolar (~10-20 μM). In addition, the extra moieties may potentially modify the effects of the cysteine targeting core as these could make additional contacts and could become important for more selective blockage of specific binary MAPK-partner protein binding events, which, however, would only be apparent in cells. Overall, these cell-based tests demonstrate that the cyclohexenone/pentenone scaffold provides a synthetically well-variable solution to produce compounds that modulate MAPK signaling network activity by influencing MAPK D-groove mediated binding.

TABLE 5 shows the MAPK binding affinity of different compounds extended at C4. K_i values, Ki_{app}, were determined in competitive protein–D-peptide binding assays with ERK2, p38α, and JNK1. (* indicates only partial inhibition, suggesting weak binding; parameter error estimate from weighted least squares method, N=3)

		Configuration	ERK2 Ki_{app}/μM	p38 Ki_{app}/μM	JNK1 Ki_{app}/μM
RU188		*R*	3.8 ± 0.2	3.5 ± 0.2	17.0 ± 2.0
RU83		*S*	1.0 ± 0.1	0.8 ± 0.6	3.4 ± 0.5
RU100		*S*	1.4 ± 0.2	1.1 ± 0.1	2.4 ± 0.3
RU101		*S*	0.8 ± 0.1	0.9 ± 0.2	1.7 ± 0.4
RU102		*S*	1.0 ± 0.1	1.3 ± 0.0	1.6 ± 0.4
RU103		*S*	2.3 ± 0.1	3.5 ± 0.9	2.4 ± 0.8
RU189		*R*	15.7 ± 2.9	12.8 ± 3.0	13.6 ± 6.1
RU115		*S*	4.4 ± 0.7	2.8 ± 0.7	8.8 ± 2.6
RU140		*R*	3.8 ± 0.2	1.8 ± 0.7	2.9 ± 1.1
RU141		*S*	1.0 ± 0.0	1.5 ± 0.3	2.7 ± 0.6
RU214		*R*	- *	3.9 ± 0.8	2.4 ± 0.9
RU215		*S*	- *	3.1 ± 0.5	1.3 ± 0.6

To elucidate how configuration and the substituent groups at key positions affect the binding mode of cyclohexenone compounds targeting the MAPK D-groove, we determined the crystal structure of three different enantiomers bound to ERK2: ERK2-RU67/68, ERK2-RU75/76, and ERK2-RU60/RU187 (**FIGURE 10**) (**TABLE 6**). High-resolution structural data (~1.6-2.0 Å) on these complexes could be summarized as the following. The stereochemical configuration around the covalent bond forming C3 stereogenic center - namely at C2 linked to the directing moiety, and C4 linked to the two sterical crowding groups - can greatly vary. Note that in addition to the existing stereogenic center at C4, new centers at C3 and C2 will naturally form upon covalent adduct formation. The concrete binding conformation will depend on the directing substituent group in C2 (e.g., *tert*-butyl or benzyl) as well as on the original configuration (e.g., *S* or *R*) and/or substituent group size (methyl or benzyl next to carboxymethyl) at C4. Moreover, the binding affinity is also affected by the substituent group at C5. RU78, which is a 5-methyl derivative of RU60, bound weaker to ERK2 (and to the other MAPKs, too; see Table 2) because this somewhat bigger cyclohexenone derivative would likely be less compatible with the binding pocket topography around the cysteine-warhead adduct. Structural comparisons between apoERK2, ERK2–D-peptide, and ERK2-cyclic warhead complexes show that in the latter case, the hydrophobic pocket area of the D-groove widens to accommodate the cyclohexenone ring at φ_A. For the RU60/RU187 enantiomeric pair, the binding mode is fundamentally different depending on the configuration of the substituent group at C4: RU60 forms a cysteine-adduct, while RU187 forms a mixture (~50-50%) of cysteine(161)- and histidine(125)-adducts in the crystal structure. This unexpected behavior of RU187 could only be explained by its different configuration which is likely not optimal in the chiral environment around cysteine(161), but it is a better fit for the nearby histidine(125), which unexpectedly is also a suitable nucleophile for the Michael-acceptor of the cyclic warhead.

The two enantiomers of RU70, namely (RU73 and RU77) showed the greatest difference in their binding affinity for all three MAPKs tested. The *R,R* versus the *S,S* stereoisomer at C4 and C6 bound to MAPKs with 8-10 fold higher affinity (see Table 2). Because of the extra connectivity between C4 and C6, the warhead is more extended perpendicular to the cyclohexenone ring. This more complex three-dimensional structure apparently is more sensitive to the configuration at C4. Noteworthy, the biggest difference between the enantiomers was detected for JNK1, which has a narrower hydrophobic pocket compared to that of ERK2 and p38α (Garai et al, 2012). Moreover, cyclohexenone-based compounds are generally bound to p38α better (see Tables 1-3 and 5). This could be explained by the more flexible overall structure of p38α (Kumar et al, 2018): the docking groove of this MAPK accommodates an optimal cysteine-warhead adduct conformation easier, and/or noncovalent contacts mediated by the directing moiety can contribute more to overall binding energy. The crystal structure of the ERK2-RU77 complex revealed an unexpected binding mode of this C4-C6 bridged compound: instead of the cysteine(161) thiol, the compound formed the covalent adduct with the imidazole sidechain of histidine(125) and the cysteine thiol remained intact (**FIGURE 11A**). Since RU77 bound to p38α with the highest affinity (see Table 2), we tested the effect of this compound on MKK6-p38α binding using the NanoBiT protein-protein interaction assay (**FIGURE 11B**). MKK6 is the activator kinase of p38α and the two proteins interact in a D-groove dependent manner (Garai et al, 2012) (Zeke eta al., 2015). This experiment showed that RU77 (used in 10 μM concentration) can block this binary interaction in live HEK293T cells.

TABLE 6 shows the details of protein crystallization, crystallographic data collection, and structure solution. (The enantiomer of RU68 is RU67, shown in Table 4; RU60/RU187 and RU75/RU76 are two other enantiomeric pairs).

	ERK2-RU68	ERK2-RU60	ERK2-RU187	ERK2-RU75	ERK2-RU76	ERK2-RU77
Crystallization conditions[a]	30% PEG1000 0.1 M Na-citrate pH 5.5	20% PEG8000 0.1 M Na-citrate pH 5.5	10% PEG8000 0.1 M Na-citrate pH 5.5	30% PEG3350 0.1 M Tris pH 8.5	20% PEG8000 0.1M HEPES pH 7.5	10% PEG8000 0.1M Tris pH 8.5
Data collection						
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions						
a, *b*, *c* (Å)	41.90, 78.33, 127.14	41.65, 76.86, 124.92	41.44, 76.84, 123.32	41.49, 77.05, 123.39	41.58, 77.15, 123.68	41.94, 78.47, 122.74
α, *β*, *γ* (°)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
Resolution range (Å)	49.36-2.55	48.47-1.9	61.66-1.95	38.52-1.65	48.25-1.8	48.34-1.80
$CC_{1/2}$[b]	0.998	0.999	0.999	0.999	0.999	1.000

	(0.636)	(0.693)	(0.567)	(0.718)	(0.780)	(0.560)
R_{merge}[c]	0.16	0.06	0.13	0.97	0.12	0.063
	(1.52)	(0.81)	(3.42)	(1.42)	(1.32)	(2.041)
$I/\sigma I$	11.8	12.8	12.3	14.0	13.4	20.3
	(1.5)	(1.7)	(0.9)	(1.4)	(1.6)	(1.4)
Completeness (%)	99.9	91.7	100	100	100	100
	(100)	(90.8)	(100)	(100)	(100)	(100)
Redundancy	11.6	4.6	13.4	12.5	12.7	13.3
	(12.1)	(3.9)	(13.7)	(12.6)	(12.4)	(13.2)
No. reflections	14259	29741	29520	48499	37729	38411
Refinement						
R_{work}/ R_{free}	0.1897 / 0.2318	0.1904 / 0.2240	0.2039 / 0.2354	0.1768 / 0.2069	0.1791 / 0.2064	0.1867/ 0.2222
No. atoms	2832	2921	2917	3340	3206	3032
Protein	2761	2738	2756	2962	2939	2810
Ligand/ion	52	46	86	81	53	70
Water	19	137	75	297	214	152
B-factors (Å^2)	70.57	52.45	48.93	38.27	44.87	44.14
Protein	70.77	52.47	49.15	37.82	44.64	43.74
Ligand/ion	63.05	58.36	46.37	39.67	58.67	59.24
Water	62.40	50.10	44.08	42.42	44.73	44.43
Ramachandran						
Favored (%)	97.32 %	96.94 %	98.18 %	97.98 %	97.69 %	97.35 %
Allowed (%)	2.68 %	3.06 %	1.82 %	2.02 %	2.31 %	2.65 %
Outliers (%)	0.00 %	0.00 %	0.00 %	0.00 %	0.00 %	0.00 %
R.m.s deviations						
Bond lengths (Å)	0.008	0.006	0.006	0.012	0.009	0.011
Bond angles (°)	1.021	0.769	0.931	1.163	1.049	1.061

[a] Crystals were grown with the vapor diffusion method and were frozen in liquid nitrogen in mother liquor supplemented with 25 % glycerol.
[b] Values in parenthesis are for the highest resolution bin.
[c] $R_{merge} = \Sigma_{hkl}\Sigma_i |I_i(hkl)-<I(hkl)>|/\Sigma_{hkl}\Sigma_i I_i(hkl)$

In contrast to "free" solvent exposed thiols, for example in glutathione, the sulfhydryl group of a cysteine from a protein domain is in a more specific chemical environment: surface topography and the chemical nature of the neighboring amino acid sidechains will affect the intrinsic capacity of the α,β-unsaturated Michael acceptor (C3) to form the covalent bond. The thiol of Cys116 in JNK1 is more open and thus is more accessible compared to the MAPK D-groove cysteine (see Figure 5A). Therefore, this latter is a better test case to analyze the intrinsic reactivity of the new cyclic warhead scaffold design, because steric factors would be less disturbing at an "open" thiol position. Cysteine reactivity of covalent warheads can be adjusted by modifying the electronic properties of the α,β- unsaturated carbonyl atom. Acrylamide-based warheads form an irreversible covalent bond with noncatalytic surface cysteines, while installing a nitrile group, for example in cyanoacrylamide based warheads, the intrinsic reactivity of the olefin towards thiols increases but the formation of the irreversible adducts is eliminated. Cyanoacrylamide based warheads had indeed been successfully applied to increase the occupancy of inhibitors originally developed to bind at the ATP-pocket of kinases (Serafimova et al, 2012)(Bradshaw et al, 2015). However, the electronic properties and/or the elimination of Michael-donor amino acid residues may also be achieved through dramatically different chemical solutions: instead of a nitrile, keto groups may also be useful for increasing intrinsic reactivity combined with steric crowding to facilitate the elimination of Michael-donor amino acid residues (which could be referred to as "frustration" via sterical crowding). Additionally, the steric shielding around the reactive center of the cycloalkenone moiety can block the formation of stable adducts with hindered amino acid residues endowing higher site selectivity for the ligands and mitigating "off-target" binding. The cyclic cyclohexenone/pentenone scaffolds provide a new platform: the electronic, steric properties, or ring strain could all be fine-tuned. As a test case, we synthesized a set of JNK inhibitors that all contained the same ATP-pocket binding moiety (IN-8) but had different warheads (Zhang et al, 2012). The warheads were the following: acrylamide – acyclic irreversible (JNK-IN-8), cyanoacrylamide (RU155) – acyclic reversible, and cyclohexenone/pentenone – cyclic reversible (BD837, BD838, RU154, RU153, and RU172). These were similarly linked to IN-8 so that the α,β- unsaturated carbonyl atom (C3) could selectively target a surface cysteine located next to the ATP-pocket (Cys116 in JNK1). In addition, we also synthesized two additional BD837-IN-8 derivatives, in which the ATP-pocket binding moiety was linked to the cyclic warhead scaffold via an additional methylene group (RU135), making the distance between these two less favorable, or this was further modified by changing the amide at C2 with an ester group (RU137; where the latter has stronger electron withdrawing capacity making the Michael acceptor, C3, more reactive). The crystal structure of the JNK1–BD837-IN-8, –BD837-IN-8, and –RU135-IN-8 complexes were

determined to reveal the structures of the Cys116 covalent adduct (**FIGURE 12** and **TABLE 7**). The kinetic binding properties of the inhibitors were measured by SPR and a 2-step reversible kinetic model was established for kinase-inhibitor binding (**FIGURE 13A**). The reversible binding scheme included the formation of the noncovalent kinase-inhibitor (KI) complex as well as the covalent adduct (KI^{cov}). The association and dissociation of KI are described by the k_1 and k_2 binding rates, while the formation of KI^{cov} from KI or the formation of KI from KI^{cov} are described by k_3 and k_4, respectively. (To determine all four parameters unambiguously based on numerical fitting the kinetic plots to the 2-step reversible kinetic model, k1 and k2 were first independently determined using the cysteine to serine mutated protein (JNK1 Cys116Ser) (**FIGURE 13B,C**). Reversible inhibitor potency ranking can be based on the steady-state inhibition constant, $K_i = (k_2/k_1) / (1+ (k_3/k_4))$ in molarity (M) (Mons et al, 2022). This analysis can also be used to calculate the energy contribution of reversible covalent bond formation for each inhibitor ($\Delta\Delta G$) (**TABLE 8**). This comparative analysis showed that the reactivity of the cyclic warhead can be tuned via its substituent groups, and these can be used to set the binding affinity and to dramatically increase residence time (i.e. decrease k_{off}) (**FIGURE 13D**).

TABLE 7 shows the details of protein crystallization, crystallographic data collection, and structure solution of JNK1–BD837-IN-8, –BD837-IN-8, and –RU135-IN-8 complexes.

	JNK1-BD837-IN-8*	JNK1-BD838-IN-8*	JNK1-RU135-IN-8*
Crystallization conditions[a]	12% PEG3000, 2% MPD, 0.1M HEPES pH 7.5	12% PEG3000, 4% MPD, 0.1M HEPES pH 7.5	8% PEG3350, 0.1 M, HEPES pH 7.5
Data collection			
Space group	$P3_1$	$P3_1$	$P3_1$
Cell dimensions			
a, *b*, *c* (Å)	106.26 106.26 99.72	106.55 106.55 99.79	107.72 107.72 99.08
α, β, γ (°)	90,90, 120	90,90, 120	90,90, 120
Resolution range (Å)	46.89-2.41	47.00- 2.70	47.32-2.78
$CC_{1/2}$[b]	0.999 (0.48)	0.999 (0.544)	0.999 (0.518)
R_{merge}[c]	0.073 (2.156)	0.102 (2.317)	0.094 (2.189)
$I/\sigma I$	16.0 (0.9)	17.0 (1.2)	18.0 (1.4)
Completeness (%)	99.8 (98.2)	99.9 (100.0)	99.9 (99.9)
Redundancy	10.5 (7.3)	10.7 (10.8)	10.8 (10.8)
No. reflections	48522	34745	32333
Refinement			
R_{work}/ R_{free}	0.2097 / 0.2262	0.2063 / 0.2256	0.2032 / 0.2179
No. atoms	8442	8391	8361
Protein	8277	8257	8215
Ligand/ion	138	132	141
Water	27	2	5
B-factors (Å^2)	84.37	89.09	93.04
Protein	84.50	89.10	92.93
Ligand/ion	79.43	88.34	99.53
Water	70.25	66.86	82.87
Ramachandran			
Favored (%)	97.03 %	96.92 %	96.00 %
Allowed (%)	2.88 %	3.08 %	4.00 %
Outliers (%)	0.10 %	0.00 %	0.00 %
R.m.s deviations			
Bond lengths (Å)	0.007	0.007	0.006
Bond angles (°)	1.048	1.035	1.005

*Twinning was present, -h,-k,l twinning operator was used during refinement. The final crystallographic models contain three JNK1-small molecule complexes in the asymmetric unit.

[a] Crystals were grown with the vapor diffusion method and were frozen in liquid nitrogen in mother liquor supplemented with 25 % glycerol.
[b] Values in parenthesis are for the highest resolution bin.
[c] $R_{merge} = \Sigma_{hkl}\Sigma_i |I_i(hkl)-\langle I(hkl)\rangle|/\Sigma_{hkl}\Sigma_i I_i(hkl)$

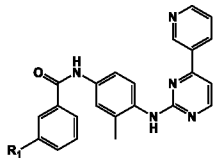
TABLE 8 shows the values of k_{1-4}, K_i $[(k_2/k_1) / (1+ (k_3 /k_4)]$, and the energy contribution of reversible bond formation ($\Delta\Delta G$). (* Not claimed, for reference only.)

	JNK-IN-8*	RU155-IN-8*	BD837-IN-8	BD838-IN-8	RU154-IN-8	RU153-IN-8	RU172-IN-8
k_1	1.5E+6	1.1E+5	1.9E+5	2.9E+5	1.9E+5	1.3E+5	2.5E+5
k_2	0.15360	0.09983	0.03172	0.05346	0.10230	0.04122	0.03529
k_3	0.02952	0.01140	0.05218	0.02464	0.00615	0.03598	0.02385
k_4	-	0.00026	0.00021	0.00029	0.00111	0.00129	0.00028
K_i (nM)	-	20.6	0.7	2.1	81.0	10.7	1.6
$\Delta\Delta G$ (kcal/mol)	-	-2.25	-3.26	-2.64	-1.11	-1.99	-2.64
	RU135-IN-8	RU137-IN-8	RU212-IN-8	RU213-IN-8	RU210-IN-8	RU211-IN-8	
k_1	3.4E+5	3.9E+5	1.64E+5	3.76E+5	3.0E+5	3.11E+5	
k_2	0.0552	0.06206	0.0346	0.0797	0.1080	0.1272	
k_3	0.60000	0.36693	0.010636	0.01045	0.05831	0.096979	
k_4	0.02831	0.00508	0.000056	0.000156	0.00117	0.004207	
K_i (nM)	7.4	2.2	1.1	3.1	7.1	17.0	
$\Delta\Delta G$ (kcal/mol)	-1.83	-2.54	-3.1	-2.5	-2.33	-1.88	

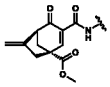
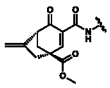
Because of the "frustration" of the α,β-unsaturated carbonyl (originating from the sterical crowding effect of the substituent groups located on the cyclohexenone/pentenone ring) and the presence of nearby strong electron withdrawing groups, covalent bond formation of composite inhibitors carrying the new cyclic Michael acceptor warhead is reversible and is resilient to millimolar amounts of GSH. We examined the inhibitory capacity of the irreversible JNK-IN-8 and the reversible BD837-IN-8 compounds after exposing them to high amounts of GSH. When these two compounds were incubated in the presence of 10 mM GSH for 6 or 18 hours before adding them into the in vitro kinase assay (PhALC), the IC_{50} of JNK-IN-8 drastically dropped but BD837-IN-8 stayed similarly "active" in blocking MAPK activity (**FIGURE 14A**). Unexpectedly, these experiments demonstrate that BD837-IN-8 stays intact even after 18 hours of incubation in 10 mM GSH, while JNK-IN-8 becomes "inactive" and its efficiency dramatically drops within a few hours. This latter is due to covalent adduct formation with GSH based on an analysis with electrospray mass spectrometry (**FIGURE 14B**). Overall, these in vitro experimental results suggest that BD837-IN-8, due to its reversible cysteine targeting and its similar potency compared to JNK-IN-8, may be a superior JNK inhibitor *in vivo*.

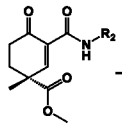
Further studies in vitro and in cell-based assays were conducted to compare reversible and irreversible JNK inhibitors. First, we used the in vitro PhALC assay (with a JNK-specific D-SENSOR) to measure the inhibitory capacity (IC_{50}) of different covalent warhead containing JNK inhibitors in the presence of 10 mM GSH. In addition, some of the inhibitors' capacity to bind to the JNK1 ATP-pocket was also assessed by the NanoBRET target engagement assay in live HEK293T cells (Anastassiadis et al., 2011). Finally, interference with c-Jun phosphorylation was monitored by quantitative western blots using the SH-SY5Y (neuroblastoma) MKK7 ACT cell line allowing specific activation of JNK upon doxycycline treatment (p-c-Jun EC_{50}). We also tested some additional covalent inhibitors using the PhALC assay. These contained varieties of the IN-8 ATP-binding moiety (IN-7, IN-9, and IN-10; (Zhang et al., 2012)) or a modified version of the IN-7 moiety in which one of the phenyl groups was replaced with a phenyl isostere, bicyclo[1.1.1]pentane, which modification was aimed to improve physicochemical and pharmacological properties (RU159-IN-7_isoPHEN) (Stepan et al., 2012). These inhibitors performed similarly or slightly better compared to BD837-IN-8 in the PhALC assay (**TABLE 9**).

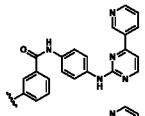
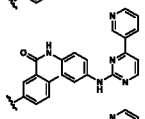
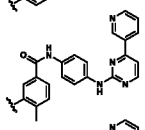
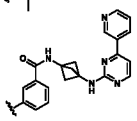
TABLE 9 shows the results of in vitro PhALC (10 mM GSH), cell-based NanoBRET, and p-cJun phosphorylation IC_{50} measurements. (* Not claimed, for reference only; [a] parameter error estimate from weighted least squares method, N=3; [b] mean of two independent measurements, N=2; [c] parameter error estimate from least squares method, N=1; n.a.: not available, not measured)



	R_1	[a]PhALC IC_{50}/(nM)	[b]NanoBRET EC_{50}/(nM)	[c]p-c-Jun EC_{50}/(nM)
IN-8		3805 ± 402	467	15800 ± 2500
JNK-IN-8		44 ± 6	12	760 ± 120[a]
RU155-IN-8		318 ± 45	192	13000 ± 2000[a]
BD837-IN-8		22 ± 6	11	660 ± 100[a]
BD838-IN-8		30 ± 5	n.a.	470 ± 200
RU144-IN-8		15 ± 3	n.a.	650 ± 20[a]
RU135-IN-8		225 ± 17	n.a.	2700 ± 600
RU137-IN-8		349 ± 24	n.a.	7000 ± 3000
RU154-IN-8		780 ± 159	75	2900 ± 1000
RU153-IN-8		254 ± 55	n.a.	1500 ± 500
RU172-IN-8		37 ± 4	8	n.a.

	R_1	[a]PhALC IC_{50}/(nM)
RU210-IN-8		641 ± 126
RU211-IN-8		537 ± 131
RU212-IN-8		59 ± 4
RU213-IN-8		128 ± 3



	R_2	[a]PhALC IC_{50}/(nM)
RU174-IN-7		17 ± 5
RU175-IN-9		12 ± 1
RU182-IN-10		13 ± 2
RU159-isoPHEN		16 ± 2

Next, we assessed the inhibitory capacity of JNK-IN-8 and BD837-IN-8 in cells on the JNK pathway. First, HEK293T cells were treated with sorbitol which is known to activate the JNK dependent signaling pathway (Bogoyevitch et al., 1995). Both inhibitors efficiently blocked JNK mediated c-Jun phosphorylation when added in 1 μM concentration but JNK phosphorylation was unaffected. Since JNK-IN-8 binding to JNK is irreversible, the electrophoretic mobility of JNK on the SDS-PAGE was changed after inhibitor binding. In contrast, BD837-IN-8 treatment did not affect the electrophoretic mobility of JNK, suggesting that the binding of this inhibitor to JNK is indeed reversible (**FIGURE 15A**). In the following experiments we used an engineered human neuroblastoma cell line (SH-SY5Y MKK7 ACT) where specific JNK pathway activation was triggered by the addition of an artificial inducer (doxycycline). This cellular system allows specific activation of JNKs while leaving other MAPK pathways unaffected (Kirsch et al., 2020). JNK-mediated c-Jun phosphorylation was examined after 2 or 6 hours of doxycycline (DOX) treatment, where longer DOX induction triggered higher JNK activity flux. The addition of the two inhibitors in 50 nM or 1 μM concentrations showed that both inhibitors block c-Jun phosphorylation similarly, albeit the reversible compound seemed to perform better (**FIGURE 15B**). The EC_{50} value of both compounds on c-Jun phosphorylation triggered by 6 hours of DOX stimulation was found to be similar (~ 0.7 μM; see Table 9 and **FIGURE 15C**), while a cyanoacrylamide containing compound (RU155-IN-8) showed weaker effect (see Table 9 and **FIGURE 15D**). Further quantitative experiments, designed to assess inhibitory capacity after short incubation, showed that BD837-IN-8 decreased c-Jun phosphorylation levels significantly more 2 hours after administration compared to JNK-IN-8, while the two inhibitors blocked c-Jun phosphorylation similarly after 4 hours (**FIGURE 15E**).

We noticed that specific JNK activation in this neuroblastoma cell line leads to decreased cell survival after 72 hours, likely initiating cell death (Le-Niculescu et al., 1999)(Zeke et al., 2016). We tested whether this JNK-specific response in this cell line could be blocked by the addition of JNK-inhibitors. It was found that reversible warhead-containing compounds (BD837/BD838-IN-8, RU159-IN7_isoPHEN) as well as the irreversible JNK-IN-8 inhibitor protected cells

from cell death to a significantly greater extent compared to IN-8 (i.e. the ATP competitive moiety alone). This showed that the reversible warhead could perform similarly to an irreversible one in this cellular setting and confirmed that covalent targeting indeed enhances the biological effect of a noncovalent directing group (IN-8) (**FIGURE 16A**). MAPKs are known to regulate gene expression by directly phosphorylating components of the AP-1 transcription factor (e.g., c-Jun, ATF or c-Fos) (Whitmarsh et al, 1996). The effect of JNK inhibitors on AP-1 mediated transcription was probed by using the AP-1 Reporter – HEK293 Recombinant Cell Line harboring an AP-1 promoter+luciferase reporter gene cassette stably integrated into the genome. Promoter activity was stimulated by the addition of phorbol 12-myristate 13-acetate (PMA) and the transcription of the reporter gene was monitored by measuring luminescence after 6 hours (where inhibitors were co-administered with PMA). According to these experiments, JNK-IN-8 showed similarly weak inhibition to IN-8 (tested at 1, 3, and 10 μM concentrations), while BD837-IN8 and RU159-IN-7_isoPHEN clearly caused stronger inhibition. Moreover, the reversible cyanoacrylamide warhead in RU155-IN-8 showed only modest inhibition compared to the different cyclohexenone based warheads of RU210-, RU212- and BD837-IN-8 (**FIGURE 16B**). The low performance of RU155-IN-8 with a cyanoacrylamide warhead in the AP-1 transcriptional assay is in good agreement with its increased cellular EC_{50} value in the p-c-Jun phosphorylation assay (see TABLE 9). However, the poor performance of the irreversible JNK-IN-8 inhibitor in the AP1-trancriptional assay (6 hours) was unexpected, since this inhibitor performed similarly to BD837-IN-8 in the more direct p-c-Jun phosphorylation assay (at 4-6 hours). This discrepancy may be due to differences in the kinetics of JNK inhibition: BD837-IN-8 appears to be more effective at shorter incubation times (e.g., at 2 hours) in the cellular c-Jun phosphorylation assay compared to JNK-IN-8 (see Figure 17C). This faster kinetics in cells in this case is likely the reason for its better efficiency in blocking a longer term JNK-related transcriptional response.

MAPK specificity of new reversible warhead containing inhibitors were next assessed by using the PhALC assay, which showed that BD837-IN-8 has close to 1000-fold specificity towards JNK1 compared to p38α or ERK2 (**FIGURE 17A**). Next, we tested the specificity of the inhibitors in cells. We used a cell line that allows p38-specific activation (HEK293T MKK6EE) and monitored MK2 substrate kinase phosphorylation with western blots using an anti-phosphoMK2 antibody (Sok et al., 2020). These experiments showed that BD837-IN-8, RU135-IN-8, and RU137-IN-8, similarly to JNK-IN-8, do not block p38-mediated MK2 phosphorylation (**FIGURE 17B**). Finally, we tested if BD837-IN-8 may target a nearby surface cysteine (Cys163) located in the docking groove of JNK by using an in vitro fluorescence polarization-based protein-peptide binding assay. BD837-IN-8 did not affect docking peptide binding, thus suggesting that the new reversible inhibitors selectively interfere with JNK by specifically targeting its unique cysteine located at the ATP binding pocket (Cys116 in JNK1) (**FIGURE 17C**).

Compared to an acrylamide based (acyclic) warhead moiety, the more complex (cyclic) structure around the α,β-unsaturated carbonyl in the cyclohexenone/pentenone scaffold could lead to more selective cysteine targeting. Therefore, we tested the performance of BD837-IN-8 on a large portion of the human kinome to assess its specificity towards JNKs. The compound was tested in 1μM concentration on the Wild Type Kinase Panel of Reaction Biology (comprised of 340 human kinases) (Anastassiadis et al., 2011)(Wang et al, 2015). BD837-IN-8 inhibited all three JNK isoforms (JNK1/2/3) as expected. In addition, this reversible compound also inhibited LIMK1 and TNK1 more than 50% (remaining activity: 38% and 44 %, respectively), but all other kinases were mostly unaffected (**FIGURE 18**). This data suggests that BD837-IN-8 is a uniquely specific JNK inhibitor. The modest inhibition of the two off-target kinases, LIMK1 and TNK1, is likely because these two kinases contain a surface cysteine in their flexible P-loop, which could be targeted by the warhead moiety, while the ATP competitive moiety is compatible with the nucleotide binding pocket of these kinases. In agreement to this, the corresponding close homologs (LIMK2 and ACK1/TNK2) that do not have a cysteine in their P-loop were far less inhibited (remaining activity: 85% and 80%, respectively). Formerly, JNK-IN-8 was profiled in 1μM concentration using KINOMEScan methodology (DiscoveryX, 442 distinct kinases) (Zhang et al., 2012). 280 kinases were common in the DiscoveryX and Reaction Biology kinase panels and the inhibitory capacity of JNK-IN-8 and BD837-IN-8 could be compared on this common human kinase set. There were 23 kinases in the JNK-IN-8 dataset that were inhibited by the irreversible, acrylamide-based inhibitor (including the three JNKs and 20 off-target kinases). Importantly, BD837-IN-8 with its reversible, cyclohexenone-based warhead inhibited none of the 20 off-target kinases (**TABLE 10**).

TABLE 10 summarizes the human kinome profiling data with BD837-IN-8. The table shows comparative data on the effect of JNK-IN-8 (Zhang et al., 2012) and BD837-IN-8 used in 1μM concentration. The table lists only those kinases that had < 50% remaining activity in the presence of the inhibitor.

Kinase	% activity JNK-IN-8[#,*]	% activity BD837-IN-8[!]	Kinase	% activity JNK-IN-8[#,*]	% activity BD837-IN-8[!]	Kinase	% activity JNK-IN-8[#,*]	% activity BD837-IN-8[!]
JNK3	0	1	FAK	11	65	CK2α2	39	99
JNK1	0	2	IRAK1	13	85	TTK	46	77
JNK2	0	7	MYLK2	13	87	MST2	48	90
PRKX	0	102	INSR	14	101	SGK3	48	106

KIT	4	69	CK1δ	18	81	ROCK2	49	92
HIPK4	4	94	p38β	20	85	…		
PDGFRβ	5	88	DYRK2	20	91	…		
MKNK2	7	106	STK17A	24	74	TNK1	60	38
CS1FR	9	89	CDK7	39	95	LMK1	74	44

([#] Data from Zhang et al., 2012; off-target kinases with < 50% remaining activity is colored in gray; [!] Data from this study; average of two experiments; N=2; [*] for reference only, not claimed)

The new cyclohexenone and -pentenone based warheads are amenable to click chemistry or Sonogashira coupling for further functionalization. To add new groups directly to the cysteine attacking warhead moiety, the ester group at C4 was extended by using azide-alkyne cycloaddition or Sonogashira coupling. First, this group was modified with propargyl alcohol (RU144-IN-8, RU145-IN-8), which were then further extended (by cycloaddition, RU160-IN-8, RU161-IN-8; or by Sonogashira coupling, RU173-IN-8). Based on the crystal structure of the JNK1–BD837-IN-8 complex, the substituent groups at C4 are directed towards the shallow and broad substrate binding pocket of JNKs (**FIGURE 19A**). A propargyl ester was well-tolerated at this position, since RU144-IN-8 showed a similar IC_{50} value compared to that of BD837-IN-8 both in the PhALC assay in vitro and in the cell-based p-c-Jun phosphorylation assay (see Table 9). Intriguingly, the JNK isoforms (JNK1/2/3) show some sequence differences by the base of the substrate binding cleft. This latter is encoded by exon 6 which differs somewhat between the JNK isoforms (see Figure 19A). Notably this ~12 amino acid long region may also differ due to the alternative splicing of exon 6, which has an a and b form (Zeke et al., 2016). In agreement to this, compounds derived from the propargyl ester derivatives and extended by azide-alkyne cyloaddition or Sonogashira coupling showed notable differences in JNK isoform specificity (measured for JNK2a2, JNK1b1 and JNKa3, referred to below as JNK1, JNK2 and JNK3, respectively). For example, JNK1 was >10-fold inhibited by one of the two stereoisomers of the propargyl ester derivative (RU144-IN-8) than JNK2, while RU160-IN-8, in which the corresponding stereoisomer was further extended by azide-alkyne cycloaddition, had a more than tenfold selectivity for JNK3 (**FIGURE 19B**) (**TABLE 11**).

TABLE 11 summarizes the results of IC_{50} measurements using the PhALC assay with different JNK isoforms.

	JNK1 (IC_{50})[#], nM	JNK2 (IC_{50})[#], nM	JNK3 (IC_{50})[#], nM	Selectivity' 1 vs. 2	Selectivity" 3 vs. 2
IN-8[*]	3805 ± 402	2043 ± 119	2878 ± 190	1	1
JNK-IN-8[*]	43 ± 6	37 ± 9	18 ± 3	1	2
BD837-IN-8	22 ± 6	116 ± 11	21 ± 6	5	6
RU144-IN-8	15 ± 3	173 ± 6	27 ± 4	**12**	6
RU145-IN-8	36 ± 4	144 ± 6	25 ± 9	4	6
RU160-IN-8	33 ± 5	158 ± 9	15 ± 5	5	**11**
RU161-IN-8	31 ± 4	71 ± 5	9 ± 2	2	9
RU173-IN-8	299 ± 25	653 ± 85	86 ± 22	2	8
RU174-IN-7	17 ± 5	68 ± 5	22 ± 5	4	3
RU159-IN-7_isoPHEN	16 ± 2	41 ± 3	19 ± 4	3	2

[#] parameter error estimate from weighted least squares method (N=3); [*] for reference only, not claimed; 'Selectivity: 1 / ((JNK2(IC_{50}) / JNK1(IC_{50})); "Selectivity: 1 / ((JNK2(IC_{50}) / JNK3(IC_{50})); Selectivity values >10 are highlighted in bold

In addition to JNK inhibitors as ATP-competitive drugs, conceptually the new compounds may also be used as JNK-specific moieites in protein targeting chimeras (PROTACs) and thus they could affect protein function by an independent mechanism, namely by lowering the cellular level of JNK. The BD837-IN-8 moiety was linked to two different ubiquitin ligase binding moieites (von Hippel-Lindau, VHL, and cereblon, CRBN, E3 ubiquitin ligase) using a PEG-linker and click chemistry-based (Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC)) approach (RU219, RU231). In addition, a different version of RU231 containing a different linker between the two functional moieities was also produced (RU232). All three compounds were effective in lowering the cellular level of JNK1 specifically in HeLa cells in a proteasome-dependent manner (by 50-65%), confirmed by the use of the MG132 proteosome blocker compound, while p38α levels remained unaffected as expected (**FIGURE 20**).

Finally, we also tested if the new cyclohexenone warhead could be used to target a cancer-associated mutation in the Ras GTPase. Formally, the Ras G12C mutant was targeted by an irreversible acrylamide moiety in the AMG-510 compound (Canon et al, 2019). We tested the noncovalent directing moiety of this composite drug alone (AMG-510 scaffold, referred to as sc_AMG-510 henceforth) and a cyclohexenone warhead containing composite molecule (RU230). These molecules were added in 200 μM concentration to three human lung cancer cell lines: A549 (with glycine 12 mutated to serine, Ras_G12S), H1792 (with glycine 12 mutated to cysteine, Ras_G12C), and LCLC-103H (with wild-type Ras, Ras_WT) and cell proliferation was followed for three days (72 hours). Both compounds affected cell viability in the Ras_WT cell line similarly but RU230 decreased cell viability more than sc_AMG-510 in the

Ras_G12C cell line, while in the Ras_G12S cell line RU230 was less efficient as expected. These results are in agreement with the notion that the cyclohexenone moiety increases the effect of sc_AMG-510 as a chemical warhead specifically for Ras_G12C (**FIGURE 21**).

Conclusion

In cyclohexenone/pentenone the Michael acceptor, namely the α,β-unsaturated carbonyl, can be embedded into a sterically more complex intramolecular environment compared to open-chain acrylamides. The more complex chemical environment of the Michael acceptor in these synthetic building blocks can be exploited in increasing the specificity of composite drugs. The Michael acceptor-based cyclic warhead can be used as a more selective covalent anchor contributing to the binding specificity of composite drugs on their own right. We showed that composite drugs containing properly substituted cycloxenone/cyclopentenone warheads and various noncovalent directing moieties have unexpected properties. The new warhead scaffold can be used as a novel modular unit and provides a practical solution for building more specific covalent drugs. Unexpectedly, we found that in addition to cysteine thiol the new cyclic warheads could target the imidazole sidechain of histidine, which is far rarer compared to cysteine targeting. The imidazole sidechain of a histidine residue located in the MAPK protein-protein interaction surface, next to cysteine thiol, is an alternative nucleophile for reversible covalent adduct formation. Nucleophile choice for Michael addition is governed by protein surface topography around the targeted residue and is determined by the substituent groups and the concrete configuration at stereogenic centers built into the cyclic warhead structure. Moreover, reversible warheads provide an alternative to the practice of using an irreversible electrophile (e.g., acrylamide), particularly if one considers that unwanted cross-reactions in cells, tissues, or in the body are likely to be more devastating if happen through irreversible covalent bond formation. The reversible nature of the new thiol targeting warheads with six, five, or four carbon-containing cyclohexenone, cyclopentenone, or cyclobutenone ring, respectively, provides several advantages over irreversible warheads for biological tool design and for drug development: resilience against GSH and tunable reactivity and residence time. The electronic properties of the unsaturated carbonyl (C3) in the cyclic warhead architecture can be tailored via a modular synthetic approach allowing the straightforward incorporation of different substituent groups. Standardized, organocatalysis-based synthetic routes allow efficient control over the configuration of substituent groups at key positions around C3. Substituent groups in C2 and/or C4 could be exploited in directing the different substituent groups towards different pockets near to the targeted nucleophilic sidechain so that to increase binding strength or other binding properties. The cyclic warhead structure allows more selective chemical bond formation with cysteines and/or with other nucleophilic sidechains (e.g., histidine) located in a specific chiral environment because covalent bond formation will naturally generate several new stereogenic centers in the vicinity of the C-S or C-N covalent bond. In contrast, stereospecific synthetic control is far less straightforward with open-chain warhead scaffolds. Protein surface topography around a protein surface residue thus can be better exploited in inhibitor design: linking the cyclic warhead to noncovalent drugs will increase the specificity of the original inhibitor through positive selection towards the target, but also due to negative selection against off-target nucleophilic amino acid sidechains. The new cyclic warheads greatly extend the toolkit for reversible covalent drug design.

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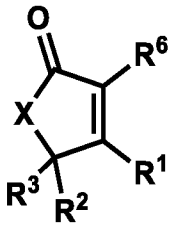
Claims

1. Compound of Formula (I)



Formula (I)

or pharmaceutically acceptable salts thereof
wherein

R^1 is H, C_{1-4} alkyl, C_{2-4} alkenyl, C_{2-4} alkynyl, C_{6-10} aryl or 5-7 membered heteroaryl or perfluorinated C_{1-4} alkyl, preferably H or C_{1-3} alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl, C_{6-10} aryl, more preferably H, C_{1-3} alkyl, or phenyl
or R^1 together with R^2 or R^3 may form a -C(O)-O-$(CH_2)_m$- group where m = 1-2, preferably 1;
or R^1 together with R^2 may form a–-$(CH_2)_n$- where n = 1-5, preferably 3-4;

R^2 is selected from C_{1-4} alkyl, C_{2-4} alkenyl, C_{2-4} alkynyl, C_{3-7} carbocyclyl, 3-7 membered heterocyclyl, C_{6-10} aryl, 5-7 membered heteroaryl, halo-C_{1-4} alkyl, aryl-C_{1-4} alkyl, -C(O)OH, -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{2-4} alkynyl, -C(O)O-C_{6-10} aryl, -C(O)O-$[(CH_2)_2O]_n$-C_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C_{2-4} alkenyl, -C(O)O-$[(CH_2)_2O]_n$-C_{2-4} alkynyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)OH, -C(O)O-$[(CH_2)_2O]_n$-C(O)OC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)O-C_{2-4} alkenyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)O-C_{2-4} alkynyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)NH_2, -C(O)O-$[(CH_2)_2O]_n$-C(O)NHC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)NC_{1-4} alkyl$_2$, -C(O)O-$[(CH_2)_2O]_n$-NH_2, -C(O)O-$[(CH_2)_2O]_n$-NHC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-NC_{1-4} alkyl$_2$, -C(O)O-$[(CH_2)_2O]_n$-C_{1-4} haloalkyl, -C(O)O-$[(CH_2)_2O]_n$-C_{6-10} aryl, -C(O)O-$[(CH_2)_2O]_n$-5-7 membered heteroaryl, -C(O)NH$(CH_2)_n$NHC(O)-C_{6-10} aryl, -C(O)NH$(CH_2)_n$NHC(O)-5-7 membered heteroaryl, -C(O)-C_{1-4} alkyl, -C(O)-C_{2-4} alkenyl, -C(O)-C_{2-4} alkynyl, -C(O)-C_{6-10} aryl, -C_{1-4} alkyl-C(O)-C_{1-4} alkyl, -C_{1-4} alkyl-C(O)-C_{1-4} alkenyl, -C_{1-4} alkyl-C(O)-C_{1-4} alkynyl, -C(O)NH_2, -C(O)NHC_{1-4} alkyl, -C(O)NHC_{2-4} alkenyl, -C(O)NHC_{2-4} alkynyl, -C(O)NC_{1-4} alkyl$_2$, -C(O)NC_{2-4} alkenyl$_2$, -C(O)NC_{2-4} alkynyl$_2$, -OH, -OC_{1-4} alkyl, -NH_2, -NHC_{1-4} alkyl, -N(C_{1-4} alkyl)$_2$, halogen, -CN, -SO_3H, -SO_3C_{1-4} alkyl, -CF_3, -C(O)O-C_{1-4} alkyl-C≡CR^7, -C(O)O-C_{1-4}-alkyl-R^8,
or together with R^5 may form a -$(CH_2)_m$-C(CH_2)- group,
where n = 1-10
m = 1-2, preferably 1,

R^3 is selected from -C(O)OH, -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{2-4} alkynyl, -C(O)O-C_{6-10} aryl, -C(O)O-$[(CH_2)_2O]_n$-C_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C_{2-4} alkenyl, -C(O)O-$[(CH_2)_2O]_n$-C_{2-4} alkynyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)OH, -C(O)O-$[(CH_2)_2O]_n$-C(O)OC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)O-C_{2-4} alkenyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)O-C_{2-4} alkynyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)NH_2, -C(O)O-$[(CH_2)_2O]_n$-C(O)NHC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)NC_{1-4} alkyl$_2$, -C(O)O-$[(CH_2)_2O]_n$-NH_2, -C(O)O-$[(CH_2)_2O]_n$-NHC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-NC_{1-4} alkyl$_2$, -C(O)O-$[(CH_2)_2O]_n$-C_{1-4} haloalkyl, -C(O)O-$[(CH_2)_2O]_n$-C_{6-10} aryl, -C(O)O-$[(CH_2)_2O]_n$-5-7 membered heteroaryl, -C(O)NH$(CH_2)_n$NHC(O)-C_{6-10} aryl, -C(O)NH$(CH_2)_n$NHC(O)-5-7 membered heteroaryl, -C(O)-C_{1-4} alkyl, -C(O)-C_{2-4} alkenyl, -C(O)-C_{2-4} alkynyl, -C(O)-C_{6-10} aryl, -C_{1-4} alkyl-C(O)-C_{1-4} alkyl, -C_{1-4} alkyl-C(O)-C_{1-4} alkenyl, -C_{1-4} alkyl-C(O)-C_{1-4} alkynyl, -C(O)NH_2, -C(O)NHC_{1-4} alkyl, -C(O)NHC_{2-4} alkenyl, -C(O)NHC_{2-4} alkynyl, -C(O)NC_{1-4} alkyl$_2$, -C(O)NC_{2-4} alkenyl$_2$, -C(O)NC_{2-4} alkynyl$_2$, -OH, -OC_{1-4} alkyl, -NH_2, -NHC_{1-4} alkyl, -N(C_{1-4} alkyl)$_2$, halogen, -CN, -SO_3H, -SO_3C_{1-4} alkyl, -CF_3, -C(O)O-C_{1-4} alkyl-C≡CR^7, -C(O)O-C_{1-4}-alkyl-R^8,
where n = 1-10 ,
or

R^2 and R^3 may be combined together with the atom to which they are attached to form a C_{4-6} carbocyclyl or 4-6 membered heterocyclyl

X is -CHR^4- or -CHR^4-CHR^5- or direct bond, preferably -CHR^4- or -CHR^4-CHR^5-,
wherein

R^4 is H, hydroxyl, C_{1-4} alkyl, C_{2-4} alkenyl,

R^5 is H, C_{1-4} alkyl, C_{2-4} alkenyl, or R^5 together with R^2 may form a–-$(CH_2)_m$-C(CH_2)- group where m = 1-2, preferably 1,

R^4 and R^5 may be combined together with the atoms to which they are attached to form a C_{3-7} carbocyclyl or 3-7 membered heterocyclyl

R^6 is C_{6-10} aryl, 5-7 membered heteroaryl, halo-C_{1-4} alkyl, aryl-C_{1-4} alkyl, -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{2-4} alkynyl, -C(O)O-C_{6-10} aryl, -C(O)-C_{1-4} alkyl, -C(O)O-C_{1-4} alkyl-C_{6-10} aryl, -C(O)-C_{2-4} alkenyl, -C(O)-C_{2-4} alkynyl, -C(O)-C_{6-10} aryl, -C(O)NH_2, -C(O)NHC_{1-4} alkyl, -C(O)NHC_{2-4} alkenyl, -C(O)NHC_{2-4} alkynyl, -C(O)NHC_{1-4} alkyl-OH, -C(O)NH-C_{6-10} aryl, -C(O)NH-C_{1-4} alkyl-C_{6-10} aryl, -C(O)NH-5-10 membered heteroaryl, -C(O)NC_{1-4} alkyl$_2$, -C(O)NC_{2-4} alkenyl$_2$, -C(O)NC_{2-4} alkynyl$_2$, -SO_3H, -SO_3C_{1-4} alkyl, -SO_3C_{6-10} aryl, -SO_2NH_2, -SO_2NHalkyl, -SO_2NHaryl, -CF_3, -C(O)O-C_{1-4} alkyl-C≡CR^7, -C(O)O-C_{1-4}-alkyl-R^8, or
R^6 is composed of 2-10 moieties selected from -C(O)-, -NH-, -N(CH_3)-, -O-, -CH_2-, optionally substituted -C_{6-10} aryl, optionally substituted 3-7 membered carbocyclyl, optionally substituted 3-7 membered heterocyclyl, optionally substituted 5-7 membered heteroaryl, resulting in a chemically reasonable radical;

R^7 is H, C_{6-10} aryl, 5-7 membered heteroaryl, vinyl, -[(CH_2)$_2$O]$_n$-C_{1-4} alkyl, -[(CH_2)$_2$O]$_n$-C_{2-4} alkenyl, -[(CH_2)$_2$O]$_n$-C_{2-4} alkynyl, -[(CH_2)$_2$O]$_n$-C(O)OH, -[(CH_2)$_2$O]$_n$-C(O)OC_{1-4} alkyl, -[(CH_2)$_2$O]$_n$-C(O)O-C_{2-4} alkenyl, -[(CH_2)$_2$O]$_n$-C(O)O-C_{2-4} alkynyl, -[(CH_2)$_2$O]$_n$-C(O)NH_2, -[(CH_2)$_2$O]$_n$-C(O)NHC_{1-4} alkyl, -[(CH_2)$_2$O]$_n$-C(O)NC_{1-4} alkyl$_2$-[(CH_2)$_2$O]$_n$-NH_2, -[(CH_2)$_2$O]$_n$-NHC_{1-4} alkyl, -[(CH_2)$_2$O]$_n$-NC_{1-4} alkyl$_2$, -[(CH_2)$_2$O]$_n$-C_{1-4} halogenoalkyl, -(CH_2)$_n$C(O)OH, -(CH_2)$_n$C(O)O-C_{1-4} alkyl, -(CH_2)$_n$C(O)O-C_{2-4} alkenyl, -(CH_2)$_n$C(O)O-C_{2-4} alkynyl, -(CH_2)$_n$C(O)NH-C_{1-4} alkyl, -(CH_2)$_n$C(O)NH-C_{2-4} alkenyl, -(CH_2)$_n$C(O)NH-C_{2-4} alkynyl, -(CH_2)$_n$C(O)N-C_{1-4} alkyl$_2$, -(CH_2)$_n$C(O)N-C_{2-4} alkenyl$_2$, -(CH_2)$_n$C(O)N-C_{2-4} alkynyl$_2$, preferably H, C_{6-10} aryl, 5-7 membered heteroaryl; where the aryl, heteroaryl are in each occurrence optionally substituted with one or more substituents independently selected from C_{1-4} alkyl, halogen, haloalkyl, hydroxyl, -OC_{1-4} alkyl, -NH_2, -NHC_{1-4} alkyl, -N(C_{1-4} alkyl)$_2$

R^8 is 5-6-membered heterocyclyl, the heterocyclyl is optionally substituted with one or more substituent independently selected from C_{1-4} alkyl, C_{2-4} alkenyl, C_{2-4} alkynyl, C_{3-10} carbocyclyl, 3-10 membered heterocyclyl, C_{6-10} aryl, 5-10 membered heteroaryl, where the carbocyclyl, heterocyclyl, aryl and heteroaryl are optionally substituted with one or more substituent independently selected from C_{1-4} alkyl, halogen, hydroxyl, -OC_{1-4} alkyl, -NH_2, -NHC_{1-4} alkyl, -N(C_{1-4} alkyl)$_2$, or 5-10 membered heteroaryl optionally substituted with 1 or 2 C_{1-2} alkyl; halo-C_{1-4} alkyl, aryl-C_{1-4} alkyl, -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{6-10} aryl, -C(O)-C_{1-4} alkyl, -C(O)-C_{2-4} alkenyl, -C(O)-C_{2-4} alkynyl, -C(O)-C_{6-10} aryl, -C(O)-C_{5-10} heteroaryl, -C(O)NH_2, -C(O)NHC_{1-4} alkyl, -C(O)NHC_{2-4} alkenyl, -C(O)NHC_{2-4} alkynyl, -C(O)NC_{1-4} alkyl$_2$, -C(O)NC_{2-4} alkenyl$_2$, -C(O)NC_{2-4} alkynyl$_2$, -CN, -SO_3H, -SO_3C_{1-4} alkyl, -CF_3, -C_{1-4} alkyl-C(O)OH, -C_{1-4} alkyl-C(O)O-C_{1-4} alkyl, -C_{1-4} alkyl-C(O)O-C_{2-4} alkenyl, -C_{1-4} alkyl-C(O)O-C_{2-4} alkynyl, -C_{1-4} alkyl-C(O)NH_2, -C_{1-4} alkyl-C(O)NHC_{1-4} alkyl, -C_{1-4} alkyl-C(O)NC_{1-4} alkyl$_2$, -[(CH_2)$_2$O]$_n$-C_{1-4} alkyl, -[(CH_2)$_2$O]$_n$-C_{2-4} alkenyl, -[(CH_2)$_2$O]$_n$-C_{2-4} alkynyl, -[(CH_2)$_2$O]$_n$-C(O)OH, -[(CH_2)$_2$O]$_n$-C(O)OC_{1-4} alkyl, -[(CH_2)$_2$O]$_n$-C(O)O-C_{2-4} alkenyl, ,-[(CH_2)$_2$O]$_n$-C(O)O-C_{2-4} alkynyl, -[(CH_2)$_2$O]$_n$-NH_2, -[(CH_2)$_2$O]$_n$-NHC_{1-4} alkyl, -[(CH_2)$_2$O]$_n$-NC_{1-4} alkyl$_2$, -[(CH_2)$_2$O]$_n$-C_{1-4}halogenoalkyl, -(CH_2)$_n$C(O)OH, -(CH_2)$_n$C(O)O-C_{1-4} alkyl, -(CH_2)$_n$C(O)O-C_{2-4} alkenyl, -(CH_2)$_n$C(O)O-C_{2-4} alkynyl, -(CH_2)$_n$C(O)NH-C_{1-4} alkyl, -(CH_2)$_n$C(O)NH-C_{2-4} alkenyl, -(CH_2)$_n$C(O)NH-C_{2-4} alkynyl, -(CH_2)$_n$C(O)N-C_{1-4} alkyl$_2$, -(CH_2)$_n$C(O)N-C_{2-4} alkenyl$_2$, -(CH_2)$_n$C(O)N-C_{2-4} alkynyl$_2$, -CH_2-C(O)NH(CH_2)$_n$NHC(O)-C_{6-10} aryl, -CH_2-C(O)O(CH_2)$_n$NHC(O)-C_{6-10} aryl, -CH_2-C(O)NH(CH_2)$_n$OC(O)-C_{6-10} aryl, -CH_2-C(O)O(CH_2)$_n$OC(O)-C_{6-10} aryl or -(CH_2)$_n$-C(O)NH-peptide where the peptide sequence consists of 1-20 amino acids, preferably 1-15 amino acids,

preferably a peptide comprising the sequence of
Xaa_{11} Xaa_{10} Xaa_9 Xaa_8 Xaa_7 Xaa_6 Xaa_5 Xaa_4 Xaa_3 Xaa_2 Xaa_1 *
where * means the *N* terminal of the peptide wherein
Xaa_{11} is an apolar residue, preferably Leu, Ile or Ala, preferably Leu,
Xaa_{10} is an apolar residue, preferably Leu, Ile or Ala, preferably Ala,
Xaa_9 is a residue comprising an amide or a basic residue, preferably Arg or Lys,
Xaa_8 is a residue comprising an amide or a basic residue, preferably Arg or Lys,
Xaa_7 is a residue comprising an amide or a basic residue, preferably Arg or Lys,
wherein at least two of Xaa_9 Xaa_8 Xaa_7 is a basic residue, preferably Arg or Lys,
Xaa_6 is an apolar residue, preferably Leu, Ile or Ala, preferably Ala,
Xaa_5 is an apolar residue, preferably Leu, Ile or Ala, preferably Leu,
Xaa_4 is a residue comprising an amide or a basic residue, preferably Arg or Lys,
Xaa_3 is a polar residue, preferably Ser or Thr,
Xaa_2 is a residue comprising an amide or a basic residue, preferably Arg or Lys,
Xaa_1 is a polar residue, preferably Ser or Thr;

preferably the following sequence: LARRRALRSKS* where * means the N terminal of the peptide

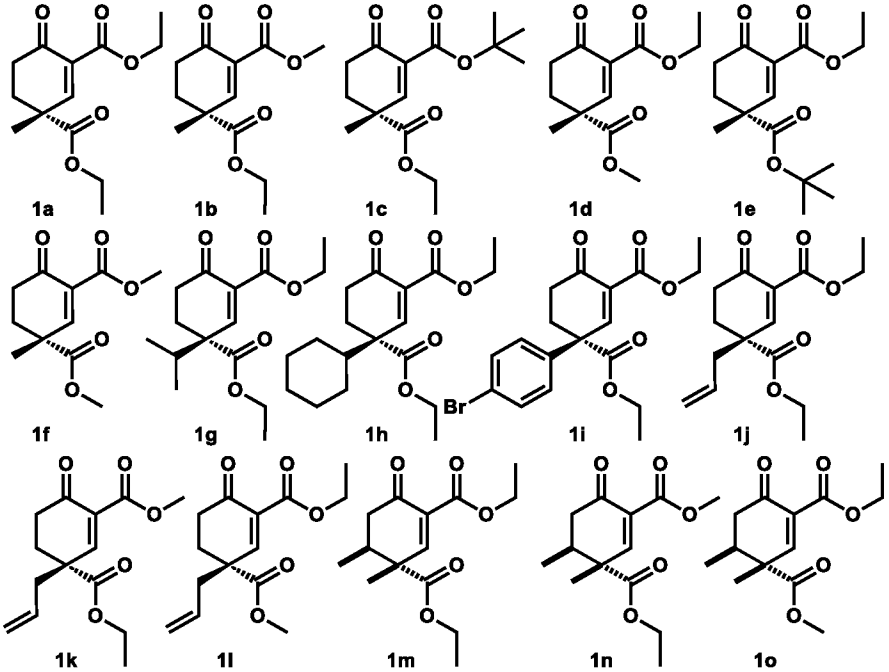
or the substituent of the 5-6-membered heterocyclyl is composed of 2-10 moieties selected from -CH_2-, -C(O)-, -NH-, -N(CH_3)-, -O-, optionally substituted -C_{6-10} aryl, optionally substituted 3-7 membered carbocyclyl, optionally substituted 3-7 membered heterocyclyl, optionally substituted 5-7 membered heteroaryl, resulting in a chemically reasonable radical;

wherein

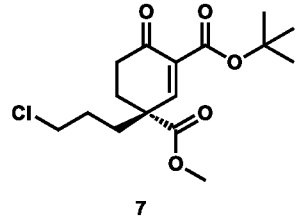
n = 1-10;

1.1 with the proviso that the following compounds are excluded from the claimed scope:

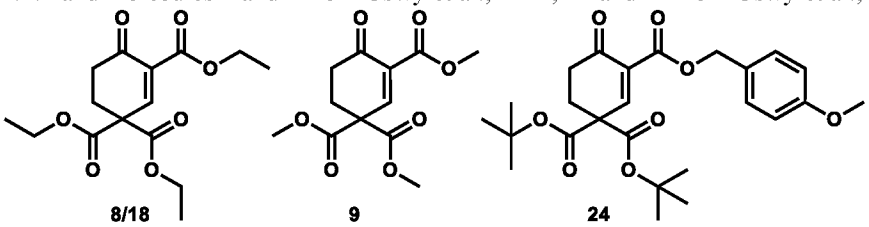
1.1.1 molecules **1a-1o** from Berkes et al., 2016



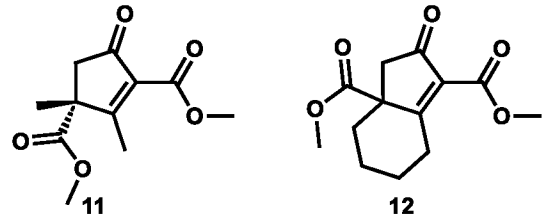
1.1.2 and molecule **7** from Varga et al., 2020



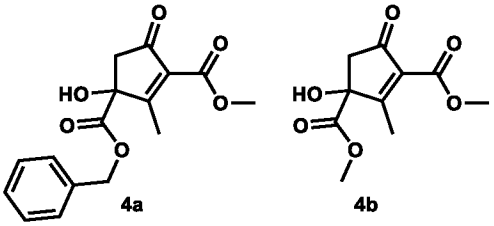
1.1.3 and molecules **8** and **9** from Oswy et al., 1994, **18** and **24** from Oswy et al., 1996



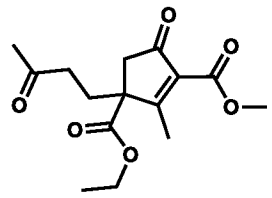
1.1.4 and molecules **11** and **12** from Kato et al., 2002



1.1.5 and molecules **4a/4b** from Okano et al., 1988

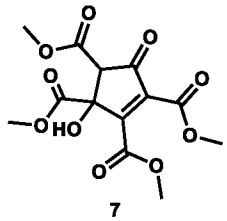


1.1.6 and molecule **9** from Dobly et al., 1968



9

1.1.7 and molecule 7 from Maier et al., 1993



7

2. Compound according to claim 1 or pharmaceutically acceptable salts thereof, wherein

R^1 is H, C_{1-4} alkyl, C_{2-4} alkenyl, C_{2-4} alkynyl, C_{6-10} aryl or 5-7 membered heteroaryl, preferably H or C_{1-3} alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl, C_{6-10} aryl, more preferably H, C_{1-3} alkyl, or phenyl;

R^2 is selected from C_{1-4} alkyl, C_{2-4} alkenyl, C_{2-4} alkynyl, C_{3-7} carbocyclyl, 3-7 membered heterocyclyl, C_{6-10} aryl, 5-7 membered heteroaryl, halo-C_{1-4} alkyl, aryl-C_{1-4} alkyl, -C(O)OH, -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{2-4} alkynyl, -C(O)O-C_{6-10} aryl, -C(O)O-$[(CH_2)_2O]_n$-C_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C_{2-4} alkenyl, -C(O)O-$[(CH_2)_2O]_n$-C_{2-4} alkynyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)OH, -C(O)O-$[(CH_2)_2O]_n$-C(O)OC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)O-C_{2-4} alkenyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)O-C_{2-4} alkynyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)NH_2, -C(O)O-$[(CH_2)_2O]_n$-C(O)NHC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)NC_{1-4} alkyl$_2$, -C(O)O-$[(CH_2)_2O]_n$-NH_2, -C(O)O-$[(CH_2)_2O]_n$-NHC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-NC_{1-4} alkyl$_2$, -C(O)O-$[(CH_2)_2O]_n$-C_{1-4} haloalkyl, -C(O)O-$[(CH_2)_2O]_n$-C_{6-10} aryl, -C(O)O-$[(CH_2)_2O]_n$-5-7 membered heteroaryl, -C(O)NH$(CH_2)_n$NHC(O) C_{6-10} aryl, -C(O)NH$(CH_2)_n$NHC(O)-5-7 membered heteroaryl, -C(O)-C_{1-4} alkyl, -C(O)-C_{2-4} alkenyl, -C(O)-C_{2-4} alkynyl, -C(O)-C_{6-10} aryl, -C_{1-4} alkyl-C(O)-C_{1-4} alkyl, -C_{1-4} alkyl-C(O)-C_{1-4} alkenyl, C_{1-4} alkyl-C(O)-C_{1-4} alkynyl, -C(O)NH_2, -C(O)NHC_{1-4} alkyl, -C(O)NHC_{2-4} alkenyl, -C(O)NHC_{2-4} alkynyl, -C(O)NC_{1-4} alkyl$_2$, -C(O)NC_{2-4} alkenyl$_2$, -C(O)NC_{2-4} alkynyl$_2$, -CN, -SO_3H, -SO_3C_{1-4} alkyl, -CF_3, -C(O)O-C_{1-4} alkyl-C≡CR^7, -C(O)O-C_{1-4}-alkyl-R^8,
or together with R^5 may form a -$(CH_2)_m$-C(CH_2)- group, where n = 1-10
m = 1-2, preferably 1,

R^3 is selected from -C(O)OH, -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{2-4} alkynyl, -C(O)O-C_{6-10} aryl, -C(O)O-$[(CH_2)_2O]_n$-C_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C_{2-4} alkenyl, -C(O)O-$[(CH_2)_2O]_n$-C_{2-4} alkynyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)OH, -C(O)O-$[(CH_2)_2O]_n$-C(O)OC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)O-C_{2-4} alkenyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)O-C_{2-4} alkynyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)NH_2, -C(O)O-$[(CH_2)_2O]_n$-C(O)NHC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)NC_{1-4} alkyl$_2$, -C(O)O-$[(CH_2)_2O]_n$-NH_2, -C(O)O-$[(CH_2)_2O]_n$-NHC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-NC_{1-4} alkyl$_2$, -C(O)O-$[(CH_2)_2O]_n$-C_{1-4} haloalkyl, -C(O)O-$[(CH_2)_2O]_n$-C_{6-10} aryl, -C(O)O-$[(CH_2)_2O]_n$-5-7 membered heteroaryl, -C(O)NH$(CH_2)_n$NHC(O) C_{6-10} aryl, -C(O)NH$(CH_2)_n$NHC(O)-5-7 membered heteroaryl, -C(O)-C_{1-4} alkyl, -C(O)-C_{2-4} alkenyl, -C(O)-C_{2-4} alkynyl, -C(O)-C_{6-10} aryl, -C_{1-4} alkyl-C(O)-C_{1-4} alkyl, -C_{1-4} alkyl-C(O)-C_{1-4} alkenyl, C_{1-4} alkyl-C(O)-C_{1-4} alkynyl, -C(O)NH_2, -C(O)NHC_{1-4} alkyl, -C(O)NHC_{2-4} alkenyl, -C(O)NHC_{2-4} alkynyl, -C(O)NC_{1-4} alkyl$_2$, -C(O)NC_{2-4} alkenyl$_2$, -C(O)NC_{2-4} alkynyl$_2$, -CN, -SO_3H, -SO_3C_{1-4} alkyl, -CF_3, -C(O)O-C_{1-4} alkyl-C≡CR^7, -C(O)O-C_{1-4}-alkyl-R^8,
where n = 1-8
or

R^2 and R^3 may be combined together with the atom to which they are attached to form a C_{4-6} carbocyclyl or 4-6 membered heterocyclyl

X is -CHR^4- or -CHR^4-CHR^5- or direct bond, preferably -CHR^4- or -CHR^4-CHR^5-,
wherein

R^4 is H, hydroxyl, C_{1-4} alkyl, C_{2-4} alkenyl,

R^5 is H, C_{1-4} alkyl, C_{2-4} alkenyl, or R^5 together with R^3 may form a –$(CH_2)_m$-C(CH_2)- group where m = 1-2, preferably 1,

R^4 and R^5 may be combined together with the atoms to which they are attached to form a C_{3-7} carbocyclyl or 3-7 membered heterocyclyl

R^6 is C_{3-7} carbocyclyl, 3-7 membered heterocyclyl, C_{6-10} aryl, 5-7 membered heteroaryl, halo-C_{1-4} alkyl, aryl-C_{1-4} alkyl, -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{2-4} alkynyl, -C(O)O-C_{6-10} aryl, -C(O)-C_{1-4} alkyl, -C(O)O-C_{1-4} alkyl-C_{6-10} aryl, -C(O)-C_{2-4} alkenyl, -C(O)-C_{2-4} alkynyl, -C(O)-C_{6-10} aryl, -C(O)NH_2, -C(O)NHC_{1-4} alkyl, -C(O)NHC_{2-4} alkenyl, -C(O)NHC_{2-4} alkynyl, -C(O)NHC_{1-4} alkyl-OH, -C(O)NH-C_{6-10} aryl, -C(O)NH-C_{1-4} alkyl-C_{6-10} aryl, -C(O)NH-5-10 membered heteroaryl, -C(O)NC_{1-4} $alkyl_2$, -C(O)NC_{2-4} $alkenyl_2$, -C(O)NC_{2-4} $alkynyl_2$, $-SO_3H$, $-SO_3C_{1-4}$ alkyl, $-SO_3C_{6-10}$ aryl, $-CF_3$, -C(O)O-C_{1-4} alkyl-C≡CR^7 , -C(O)O-C_{1-4}-alkyl-R^8, or
R^6 is composed of 2-10 moieties selected from -C(O)-, -NH-, -N(CH_3)-, -O-, $-CH_2-$ optionally substituted -C_{6-10} aryl, optionally substituted 3-7 membered carbocyclyl, optionally substituted 3-7 membered heterocyclyl, optionally substituted 5-7 membered heteroaryl, resulting in a chemically reasonable radical;

R^7 is H, C_{6-10} aryl, 5-7 membered heteroaryl, vinyl, -$[(CH_2)_2O]_n$-C_{1-4} alkyl, -$[(CH_2)_2O]_n$-C_{2-4} alkenyl, -$[(CH_2)_2O]_n$-C_{2-4} alkynyl, -$[(CH_2)_2O]_n$-C(O)OH, -$[(CH_2)_2O]_n$-C(O)OC_{1-4} alkyl, -$[(CH_2)_2O]_n$-C(O)O-C_{2-4} alkenyl, -$[(CH_2)_2O]_n$-C(O)O-C_{2-4} alkynyl, -$[(CH_2)_2O]_n$-C(O)NH_2, -$[(CH_2)_2O]_n$-C(O)NHC_{1-4} alkyl, -$[(CH_2)_2O]_n$-C(O)NC_{1-4} $alkyl_2$-$[(CH_2)_2O]_n$-NH_2, -$[(CH_2)_2O]_n$-NHC_{1-4} alkyl, -$[(CH_2)_2O]_n$-NC_{1-4} $alkyl_2$, -$[(CH_2)_2O]_n$-C_{1-4} halogenoalkyl, -$(CH_2)_n$C(O)OH, -$(CH_2)_n$C(O)O-C_{1-4} alkyl, -$(CH_2)_n$C(O)O-C_{2-4} alkenyl, -$(CH_2)_n$C(O)O-C_{2-4} alkynyl, -$(CH_2)_n$C(O)NH-C_{1-4} alkyl, -$(CH_2)_n$C(O)NH-C_{2-4} alkenyl, -$(CH_2)_n$C(O)NH-C_{2-4} alkynyl, -$(CH_2)_n$C(O)N-C_{1-4} $alkyl_2$, -$(CH_2)_n$C(O)N-C_{2-4} $alkenyl_2$, -$(CH_2)_n$C(O)N-C_{2-4} $alkynyl_2$, preferably H, C_{6-10} aryl, 5-7 membered heteroaryl; where the aryl, heteroaryl are in each occurrence optionally substituted with one or more substituents independently selected from C_{1-4} alkyl, halogen, hydroxyl, -OC_{1-4} alkyl, $-NH_2$, -NHC_{1-4} alkyl, -N(C_{1-4} alkyl)$_2$

R^8 is 5-6-membered heterocyclyl, the heterocyclyl is optionally substituted with one or more substituent independently selected from C_{1-4} alkyl, C_{2-4} alkenyl, C_{2-4} alkynyl, C_{3-10} carbocyclyl, 3-10 membered heterocyclyl, C_{6-10} aryl, 5-10 membered heteroaryl, the carbocyclyl, heterocyclyl, aryl and heteroaryl are optionally substituted with one or more substituent independently selected from C_{1-4} alkyl, halogen, hydroxyl, -OC_{1-4} alkyl, $-NH_2$, -NHC_{1-4} alkyl, -N(C_{1-4} alkyl)$_2$or 5-10 membered heteroaryl; halo-C_{1-4} alkyl, aryl-C_{1-4} alkyl, -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{6-10} aryl, -C(O)-C_{1-4} alkyl, -C(O)-C_{2-4} alkenyl, -C(O)-C_{2-4} alkynyl, -C(O)-C_{6-10} aryl, -C(O)-C_{5-10} heteroaryl, -C(O)NH_2, -C(O)NHC_{1-4} alkyl, -C(O)NHC_{2-4} alkenyl, -C(O)NHC_{2-4} alkynyl, -C(O)NC_{1-4} $alkyl_2$, -C(O)NC_{2-4} $alkenyl_2$, -C(O)NC_{2-4} $alkynyl_2$, -CN, $-SO_3H$, $-SO_3C_{1-4}$ alkyl, $-CF_3$, -C_{1-4} alkyl-C(O)OH, -C_{1-4} alkyl-C(O)O-C_{1-4} alkyl, -C_{1-4} alkyl-C(O)O-C_{2-4}alkenyl, -C_{1-4} alkyl-C(O)O-C_{2-4}alkynyl, -C_{1-4}alkyl-C(O)NH_2, -C_{1-4} alkyl-C(O)NHC_{1-4} alkyl, -C_{1-4} alkyl-C(O)NC_{1-4} $alkyl_2$, -$[(CH_2)_2O]_n$-C_{1-4} alkyl, -$[(CH_2)_2O]_n$-C_{2-4} alkenyl, -$[(CH_2)_2O]_n$-C_{2-4} alkynyl, -$[(CH_2)_2O]_n$-C(O)OH, -$[(CH_2)_2O]_n$-C(O)OC_{1-4} alkyl, -$[(CH_2)_2O]_n$-C(O)O-C_{2-4} alkenyl, -$[(CH_2)_2O]_n$-C(O)O-C_{2-4} alkynyl, -$[(CH_2)_2O]_n$-NH_2, -$[(CH_2)_2O]_n$-NHC_{1-4} alkyl, -$[(CH_2)_2O]_n$-NC_{1-4} $alkyl_2$, -$[(CH_2)_2O]_n$-C_{1-4} halogenoalkyl, -$(CH_2)_n$C(O)OH, -$(CH_2)_n$C(O)O-C_{1-4} alkyl, -$(CH_2)_n$C(O)O-C_{2-4} alkenyl, -$(CH_2)_n$C(O)O-C_{2-4} alkynyl, -$(CH_2)_n$C(O)NH-C_{1-4} alkyl, -$(CH_2)_n$C(O)NH-C_{2-4} alkenyl, -$(CH_2)_n$C(O)NH-C_{2-4} alkynyl, -$(CH_2)_n$C(O)N-C_{1-4} $alkyl_2$, -$(CH_2)_n$C(O)N-C_{2-4} $alkenyl_2$, -$(CH_2)_n$C(O)N-C_{2-4} $alkynyl_2$, -CH_2-C(O)NH$(CH_2)_n$NHC(O)-C_{6-10} aryl, -CH_2-C(O)O$(CH_2)_n$NHC(O)-C_{6-10} aryl, -CH_2-C(O)NH$(CH_2)_n$OC(O)-C_{6-10} aryl, -CH_2-C(O)O$(CH_2)_n$OC(O)-C_{6-10} aryl
or -$(CH_2)_n$-C(O)NH-peptide where the peptide sequence consists of 1-13 amino acids,
wherein
n = 1-8,
preferably a sequence of
Xaa_{11} Xaa_{10} Xaa_9 Xaa_8 Xaa_7 Xaa_6 Xaa_5 Xaa_4 Xaa_3 Xaa_2 Xaa_1 *
where * means the *N* terminal of the peptide wherein
Xaa_{11} is Leu, Ile or Ala, preferably Leu,
Xaa_{10} is Leu, Ile or Ala, preferably Ala,
Xaa_9 is Arg or Lys,
Xaa_8 is Arg or Lys,
Xaa_7 is Arg or Lys,
Xaa_6 is Leu, Ile or Ala, preferably Ala,
Xaa_5 is Leu, Ile or Ala, preferably Leu,
Xaa_4 is Arg or Lys,
Xaa_3 is Ser or Thr,
Xaa_2 is Arg or Lys,
Xaa_1 is Ser or Thr;
preferably the following sequence: LARRRALRSKS* where * means the N terminal of the peptide.

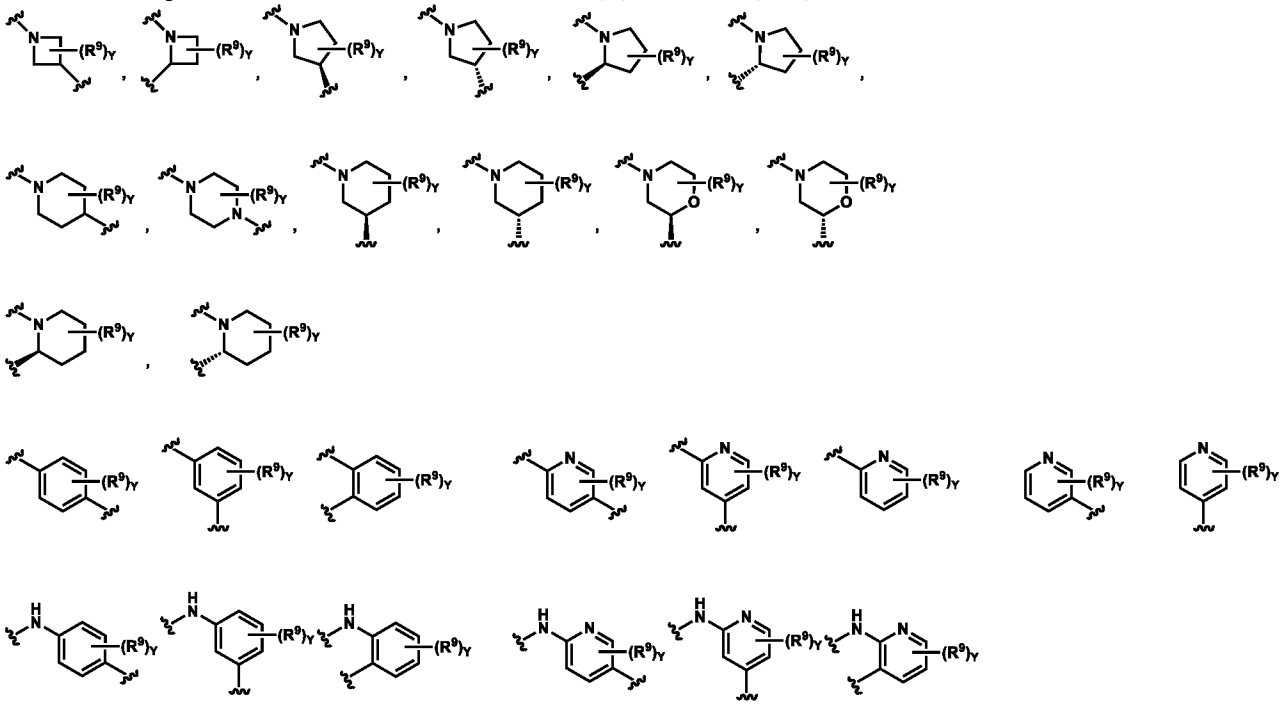
3. Compound according to claim 1 or 2 or pharmaceutically acceptable salts thereof, wherein

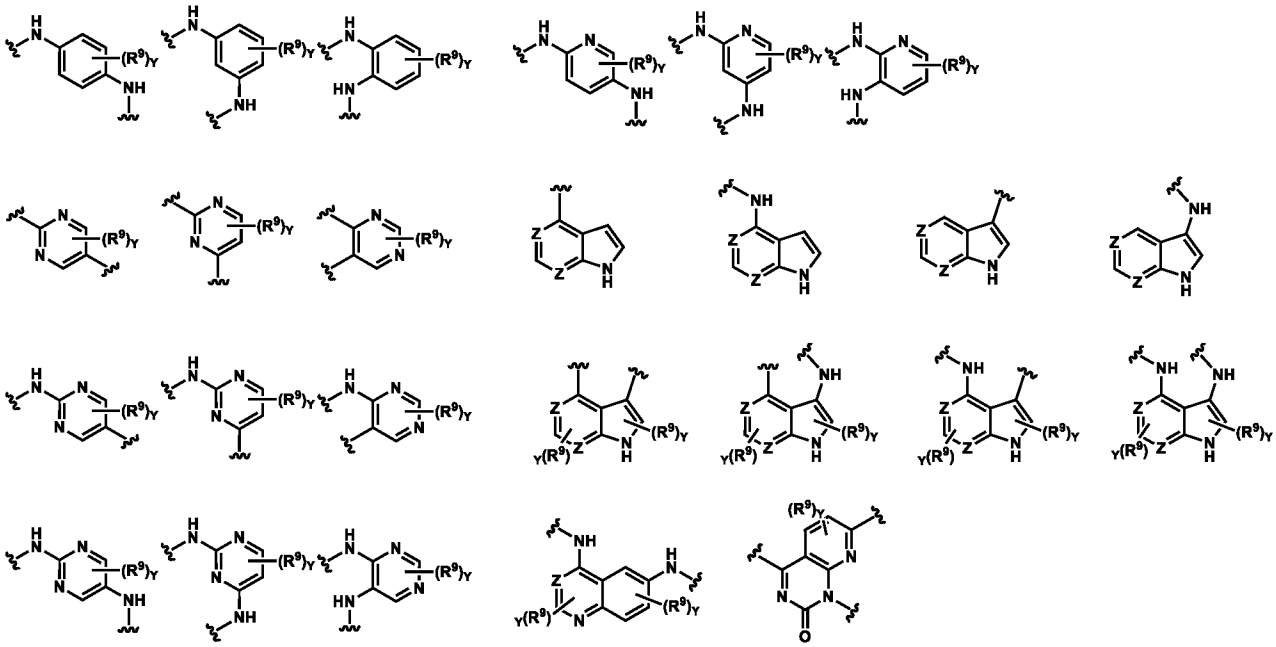
R^1 is H, C_{1-4} alkyl, C_{6-10} aryl, preferably H, C_{1-3} alkyl, phenyl, most preferably H

R^2 is selected from C_{1-4} alkyl, C_{2-4} alkenyl, C_{2-4} alkynyl, C_{3-7} carbocyclyl, 3-7 membered heterocyclyl, C_{6-10} aryl, 5-7 membered heteroaryl, halo-C_{1-4} alkyl, aryl-C_{1-4} alkyl, -C(O)OH, -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{2-4} alkynyl, -C(O)O-C_{6-10} aryl, -C(O)O-$[(CH_2)_2O]_n$-C(O)OH, -C(O)O-$[(CH_2)_2O]_n$-C(O)OC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)NH_2, -C(O)O-$[(CH_2)_2O]_n$-C(O) NHC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C(O) NC_{1-4} alkyl$_2$, -C(O)O-$[(CH_2)_2O]_n$-NH_2, -C(O)O-$[(CH_2)_2O]_n$-NHC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-NC_{1-4} alkyl$_2$, -C(O)O-$[(CH_2)_2O]_n$- C_{6-10} aryl, -C(O)O-$[(CH_2)_2O]_n$-5-7 membered heteroaryl, -C(O)O-$[(CH_2)_2O]_n$-C_{1-4} alkynyl, -C(O)NH$(CH_2)_n$NHC(O) C_{6-10} aryl, -C(O)NH$(CH_2)_n$NHC(O)-5-7 membered heteroaryl, -C(O)-C_{1-4} alkyl, -C(O)-C_{2-4} alkenyl, -C(O)-C_{2-4} alkynyl, -C(O)-C_{6-10} aryl, -C(O)O-C_{1-4} alkyl-C≡CR^7, -C(O)O-C_{1-4}-alkyl-R^8, or together with R^5 may form a -$(CH_2)_m$-$C(CH_2)$- group, wherein
n = 1-6
m = 1-2, preferably 1;

R^3 is selected from -C(O)OH, -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{2-4} alkynyl, -C(O)O-C_{6-10} aryl, -C(O)O-$[(CH_2)_2O]_n$-C(O)OH, -C(O)O-$[(CH_2)_2O]_n$-C(O)OC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C(O)NH_2, -C(O)O-$[(CH_2)_2O]_n$-C(O) NHC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-C(O) NC_{1-4} alkyl$_2$, -C(O)O-$[(CH_2)_2O]_n$-NH_2, -C(O)O-$[(CH_2)_2O]_n$-NHC_{1-4} alkyl, -C(O)O-$[(CH_2)_2O]_n$-NC_{1-4} alkyl$_2$, -C(O)O-$[(CH_2)_2O]_n$- C_{6-10} aryl, -C(O)O-$[(CH_2)_2O]_n$-5-7 membered heteroaryl, -C(O)O-$[(CH_2)_2O]_n$-C_{1-4} alkynyl, -C(O)NH$(CH_2)_n$NHC(O) C_{6-10} aryl, -C(O)NH$(CH_2)_n$NHC(O)-5-7 membered heteroaryl, -C(O)-C_{1-4} alkyl, -C(O)-C_{2-4} alkenyl, -C(O)-C_{2-4} alkynyl, -C(O)-C_{6-10} aryl, -C(O)O-C_{1-4} alkyl-C≡CR^7, -C(O)O-C_{1-4}-alkyl-R^8, wherein
n = 1-6

R^6 is 3-7 membered heterocyclyl, C_{6-10} aryl, 5-7 membered heteroaryl, -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{2-4} alkynyl, -C(O)O-C_{6-10} aryl, -C(O)O-C_{1-4} alkyl-C_{6-10} aryl, -C(O)NH_2, -C(O)NHC_{1-4} alkyl, -C(O)NHC_{2-4} alkenyl, -C(O)NHC_{2-4} alkynyl, -C(O)NC_{1-4} alkyl$_2$, -C(O)NC_{2-4} alkenyl$_2$, -C(O)NC_{2-4} alkynyl$_2$, -C(O)NHC_{1-4} alkyl-OH, -C(O)NH-C_{1-4} alkyl-C_{6-10} aryl, -C(O)NH-5-10 membered heteroaryl, -C(O)O-C_{1-4} alkyl-C≡CR^7, -C(O)O-C_{1-4}-alkyl-R^8, or
R^6 is composed of 2-10 moieties selected from -C(O)-, -NH-, -N(CH_3)-, -O-, -CH_2-,





wherein

R^9 is -OH, $-NH_2$, $-OC_{1-4}$ Alkyl, $-C_{1-4}$ Alkyl, Halogen, -CN

Y is 0-3, preferably 0-1

Z is CH, N or NH as valency permits

X is $-CHR^4-$ or $-CHR^4-CHR^5-$ or direct bond, preferably $-CHR^4-CHR^5-$

wherein

R^4 is H, C_{1-3} alkyl, C_{2-4} alkenyl, preferably H or C_{1-3} alkyl

R^5 is H, C_{1-3} alkyl, C_{2-4} alkenyl, or R^5 together with R^3 may form a $-CH_2-C(CH_2)-$ group

R^7 is H, C_{6-10} aryl, 5-7 membered heteroaryl, the aryl and heteroaryl are optionally substituted with one or more substituent independently selected from C_{1-4} alkyl, halogen, $-NH_2$, $-NHC_{1-4}$ alkyl, $-N(C_{1-4}$ alkyl$)_2$; vinyl, $-[(CH_2)_2O]_n-C(O)OH$, $-[(CH_2)_2O]_n-C(O)OC_{1-4}$ alkyl, $-[(CH_2)_2O]_n-C(O)NH_2$, $-[(CH_2)_2O]_n-C(O)NHC_{1-4}$ alkyl, $-[(CH_2)_2O]_n-C(O)NC_{1-4}$ alkyl$_2$-$[(CH_2)_2O]_n-NH_2$, $-[(CH_2)_2O]_n-NHC_{1-4}$ alkyl, $-[(CH_2)_2O]_n-NC_{1-4}$ alkyl$_2$, $-C(O)O-[(CH_2)_2O]_n-$ C_{6-10} aryl, $-C(O)O-[(CH_2)_2O]_n$-5-7 membered heteroaryl, $-C(O)NH(CH_2)_nNHC(O)$ C_{6-10} aryl, $-C(O)NH(CH_2)_nNHC(O)$-5-7 membered heteroaryl, preferably H, C_{6-10} aryl, 5-7 membered heteroaryl

R^8 is 5-6-membered heterocyclyl, the heterocyclyl is optionally substituted with one or more substituent independently selected from C_{1-4} alkyl, C_{2-4} alkenyl, C_{2-4} alkynyl, C_{3-6} carbocyclyl, 3-6 membered heterocyclyl, C_{6-8} aryl, 5-10 membered heteroaryl, where the carbocyclyl, heterocyclyl, aryl and heteroaryl are optionally substituted with one or more substituent independently selected from C_{1-4} alkyl, halogen, hydroxyl, $-OC_{1-4}$ alkyl, $-NH_2$, $-NHC_{1-4}$ alkyl, $-N(C_{1-4}$ alkyl$)_2$ or 5-10 membered heteroaryl;

halo-C_{1-4} alkyl, aryl-C_{1-4} alkyl, $-C(O)O-C_{1-4}$ alkyl, $-C(O)O-C_{2-4}$ alkenyl, $-C(O)O-C_{6-10}$ aryl, $-C(O)-C_{1-4}$ alkyl, $-C(O)-C_{2-4}$ alkenyl, $-C(O)-C_{2-4}$ alkynyl, $-C(O)-C_{6-10}$ aryl, $-C(O)NH_2$, $-C(O)NHC_{1-4}$ alkyl, $-C(O)NHC_{2-4}$ alkenyl, $-C(O)NHC_{2-4}$ alkynyl, $-C(O)NC_{1-4}$ alkyl$_2$, $-C(O)NC_{2-4}$ alkenyl$_2$, $-C(O)NC_{2-4}$ alkynyl$_2$, -CN, $-SO_3H$, $-SO_3C_{1-4}$ alkyl, $-CF_3$, $-C_{1-4}$ alkyl-C(O)O-C_{1-4} alkyl, $-C_{1-4}$ alkyl-C(O)OH, $-C_{1-4}$ alkyl-C(O)O-C_{2-4}alkenyl, $-C_{1-4}$alkyl-$C(O)NH_2$, $-C_{1-4}$ alkyl-$C(O)NH_2$, $-C_{1-4}$ alkyl-$C(O)NHC_{1-4}$ alkyl, $-C_{1-4}$ alkyl-$C(O)NC_{1-4}$ alkyl$_2$, $-[(CH_2)_2O]_n-C(O)OH$, $-[(CH_2)_2O]_n-C(O)OC_{1-4}$ alkyl, $-[(CH_2)_2O]_n-NH_2$, $-[(CH_2)_2O]_n-NHC_{1-4}$ alkyl, $-[(CH_2)_2O]_n-NC_{1-4}$ alkyl$_2$, $-CH_2-C(O)NH(CH_2)_nNHC(O)-$ C_{6-10} aryl, $-CH_2-C(O)O(CH_2)_nNHC(O)-C_{6-10}$ aryl, $-CH_2-C(O)NH(CH_2)_nOC(O)-C_{6-10}$ aryl, $-CH_2-C(O)O(CH_2)_nOC(O)-C_{6-10}$ aryl, $C(O)NH(CH_2)_nNHC(O)$-5-7 membered heteroaryl

or $-(CH_2)_n-C(O)NH$-peptide where the peptide sequence consists of 1-12 amino acids, preferably the following sequence: LARRRALRSKS* where * means the N terminal of the peptide

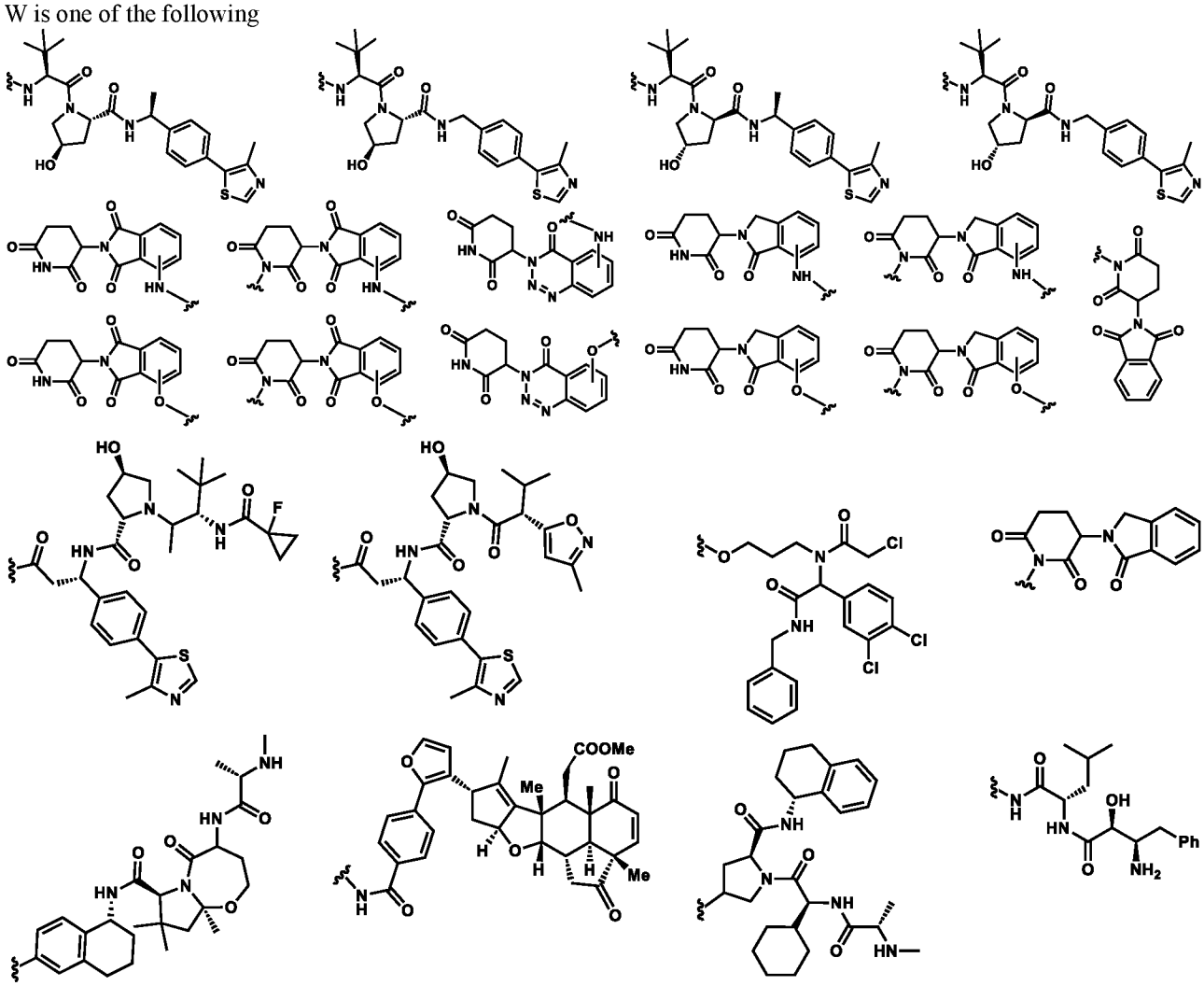
wherein

n = 1-6; or

R^8 is 5-6-membered heterocyclyl, the heterocyclyl is optionally substituted with $-[(CH_2)_2O]_n-C(O)W$, $-[(CH_2)_2O]_nNHC(O)CH_2W$, $-CH_2C(O)NH(CH_2)_nC(O)W$ wherein

n = 1-6 and

W is one of the following



4. Compound according to any of the previous claims or pharmaceutically acceptable salts thereof, wherein R^1 is H or methyl, C_6 aryl, preferably H.

5. Compound according to any of the previous claims or pharmaceutically acceptable salts thereof, wherein R^2 is selected from C_{1-3} alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl; carbocycle selected from C_{3-6} cycloalkyl groups, preferably cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl; heterocycle selected from 3-7 membered heterocyclyl, preferably 4-6 membered heterocyclyl, more preferably azetidinyl, oxetanyl, pyrrolidinyl, tetrahydrofuranyl, piperidinyl, pyranyl, morpholinyl, oxazinyl, dioxanyl; C_{6-10} aryl, 5-7 membered heteroaryl, halo-C_{1-3}alkyl, C_{6-10} aryl-C_{1-3}alkyl; -C(O)O-C_{1-4}alkyl, -C(O)O-C_{2-3}alkenyl, -C(O)O-C_{1-4} alkyl-C≡CR^7, -C(O)O-C_{1-3} alkyl-R^8, -C(O)O-C_{6-10} aryl, -C(O)-C_{1-3} alkyl, -C(O)-C_{2-3} alkenyl, -C(O)-C_{2-3} alkynyl, -C(O)-C_6 aryl,
or together with R^5 may form a -CH_2-C(CH_2)- group,

R^3 is selected from -C(O)O-C_{1-4}alkyl, -C(O)O-C_{2-3}alkenyl, -C(O)O-C_{1-4} alkyl-C≡CR^7, -C(O)O-C_{1-3} alkyl-R^8, -C(O)O-C_{6-10} aryl, -C(O)-C_{1-3} alkyl, -C(O)-C_{2-3} alkenyl, -C(O)-C_{2-3} alkynyl, -C(O)-C_6 aryl,
and

R^7 is H, C_{6-8} aryl, 5-6 membered heteroaryl, the aryl, heteroaryl optionally substituted with one or more substituents independently selected from C_{1-2} alkyl, halogen, hydroxyl, -OC_{1-4} alkyl, -NH_2, -NHC_{1-2} alkyl, -N(C_{1-2} alkyl)$_2$

R^8 is 5-6-membered heteroaryl, preferably the heteroaryl comprises 1-3 N atoms, the heteroaryl is optionally substituted with C_6 aryl, the aryl is optionally substituted with one or more substituent independently selected from C_{1-2} alkyl, halogen, hydroxyl, -OC_{1-4} alkyl, -NH_2, -NHC_{1-2} alkyl, -N(C_{1-2} alkyl)$_2$ or 5-10 membered heteroaryl; C_{1-3} alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl, halo-C_{1-3}alkyl, -C(O)NH_2, -C(O)NHC_{1-3} alkyl, -C(O)NC_{1-3} alkyl$_2$, -C_{1-3} alkyl-C(O)O-C_{1-3} alkyl, -C_{1-3} alkyl-C(O)OH, -C_{1-3} alkyl-C(O)O-C_{2-3} alkenyl, -C_{1-3} alkyl-C(O)NH_2, -C_{1-3} alkyl-C(O)NHC_{1-3} alkyl, -C_{1-3} alkyl-

$C(O)NC_{1-3}$ alkyl$_2$ or -$(CH_2)_n$-C(O)NH-peptide where the peptide sequence consists of 1-11 amino acids, preferably the following sequence: LARRRALRSKS* where * means the N terminal of the peptide.

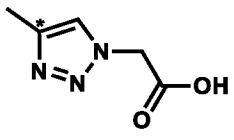
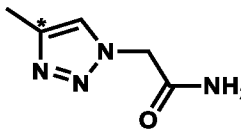
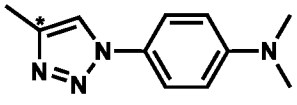
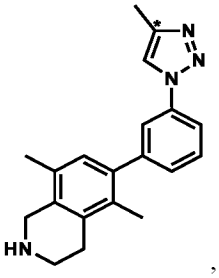
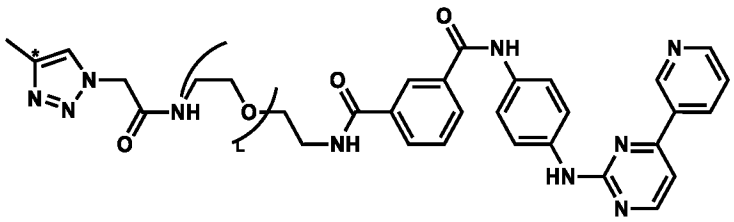
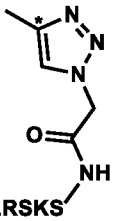
6. Compound according to any of the previous claims or pharmaceutically acceptable salts thereof, wherein

R^2 is selected from methyl, cyclohexyl, phenyl, -CH_2-phenyl, halo-C_{1-3} alkyl, -CH_2-CH_2-CH_2Cl, -CH_2-CH=CH_2, 4-Br-phenyl, -C(O)O-methyl, -C(O)O-ethyl, -C(O)O-*tert*butyl, -C(O)-phenyl, -C(O)O-CH_2-C≡CR^7, -C(O)O-$(CH_2)_2$-C≡CR^7, -C(O)O-$(CH_2)_3$-C≡CR^7, -C(O)O-$(CH_2)_4$-C≡CR^7, or -C(O)O-CH_2-R^8 ,

R^3 is selected from -C(O)O-methyl, -C(O)O-ethyl, -C(O)O-*tert*butyl, -C(O)-phenyl, -C(O)O-CH_2-C≡CR^7, -C(O)O-$(CH_2)_2$-C≡CR^7, -C(O)O-$(CH_2)_3$-C≡CR^7, -C(O)O-$(CH_2)_4$-C≡CR^7, or -C(O)O-CH_2-R^8

wherein

R^7 is H, C_{6-10} aryl, 5-6 membered heteroaryl, the aryl, heteroaryl optionally substituted with one or more substituents independently selected from -NH_2, -NHC_{1-2} alkyl, -N(C_{1-2} alkyl)$_2$,

R^8 is  ,  ,  ,  ,  ,  , LARRRALRSKS

where * is the point of attachment and

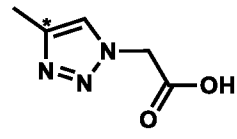
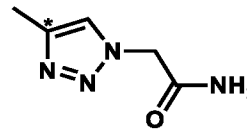
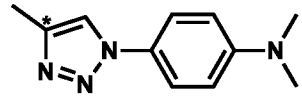
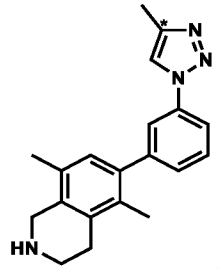
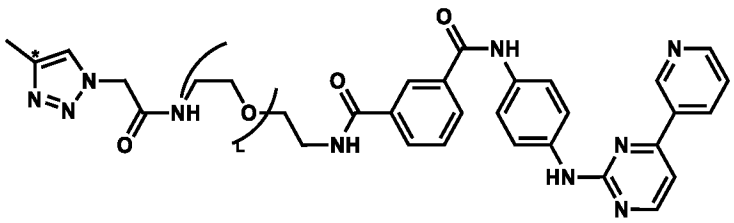
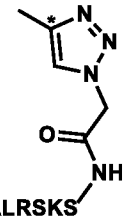
L = 1-6

or R^2 together with R^5 may form a -CH_2-C(CH_2)- group.

7. Compound according to any of the previous claims or pharmaceutically acceptable salts thereof, wherein

R^2 is C_{1-3} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{3-6} carbocyclyl, 3-6 membered heterocyclyl, C_6 aryl, 5-7 membered heteroaryl, halo-C_{1-3} alkyl, aryl-C_{1-3} alkyl, or together with R^5 may form a -CH_2-C(CH_2)- group

R^3 is -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{2-4} alkynyl, -C(O)O-C_{6-10} aryl, -C(O)-C_{1-3} alkyl, -C(O)-C_{2-4} alkenyl, -C(O)-C_{2-4} alkynyl, -C(O)-C_{6-10} aryl, -C(O)NH_2, -C(O)O-C_{1-4} alkyl-C≡CR^7, -C(O)O-C_{1-4} alkyl-R^8, wherein R^7 is H, C_{6-10} aryl, 5-6 membered heteroaryl, preferably H, C_{6-10} aryl, the aryl is optionally substituted with one or more substituent independently selected from C_{1-2} alkyl, halogen, hydroxyl, -NH_2, -NHC_{1-4} alkyl, -N(C_{1-4} alkyl)$_2$;

and R^8 is  ,  ,  ,  ,  ,  , LARRRALRSKS where * is the point of attachment and

L = 1-3

X is -CHR[4]- or -CHR[4]-CHR[5]-,
wherein
R[4] is H, methyl,

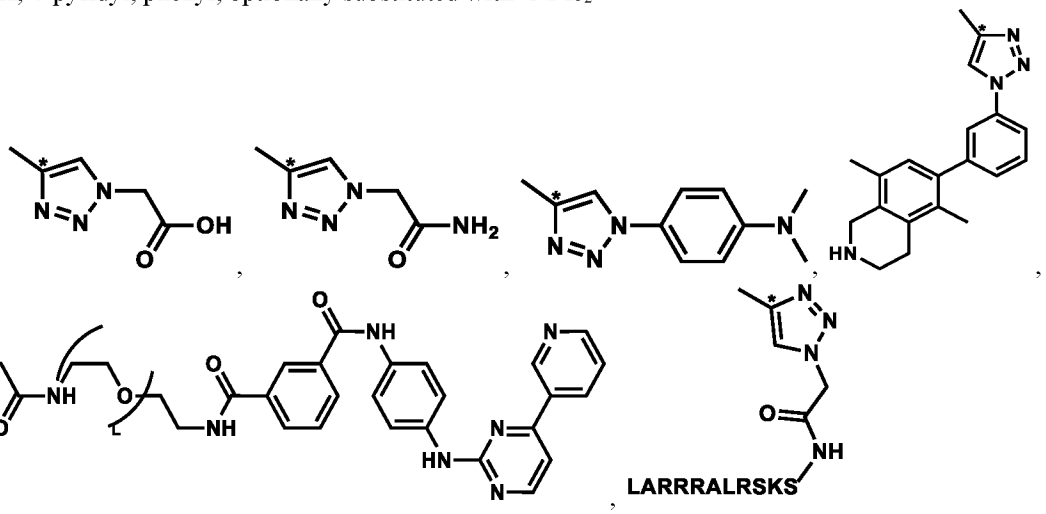
R[5] is H, or together with R[2] may form a -CH$_2$-C(CH$_2$)- group

8. Compound according to any of the previous claims or pharmaceutically acceptable salts thereof, wherein

R[2] is methyl, cyclohexyl, phenyl, -CH$_2$-phenyl, -CH$_2$-CH$_2$-CH$_2$Cl, -CH$_2$-CH=CH$_2$, 4-Br-phenyl, or together with R[5] may form a -CH$_2$-C(CH$_2$)- group, and

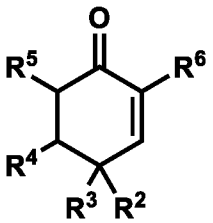
R[3] is -C(O)O-methyl, -C(O)O-ethyl, -C(O)O-*tert*-butyl, -C(O)-phenyl, -C(O)O-CH$_2$-C≡CR[7], or -C(O)O-CH$_2$-R[8],
wherein

R[7] is H, 4-pyridyl, phenyl, optionally substituted with -NMe$_2$

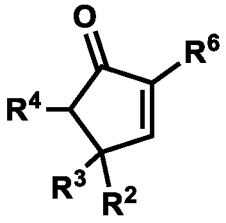
R[8] is 

where * is the point of attachment and
L = 1-3.

9. Compound according to any of the previous claims or pharmaceutically acceptable salts thereof, wherein the compound of formula (I) is a compound of formula (I-1) or formula (I-2)



Formula (I-1)



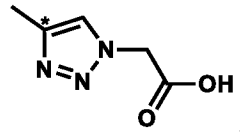
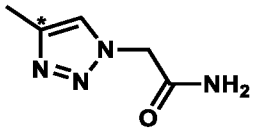
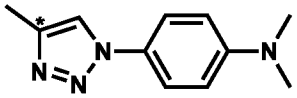
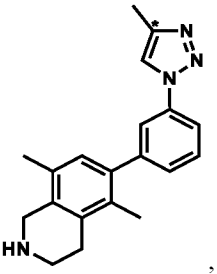
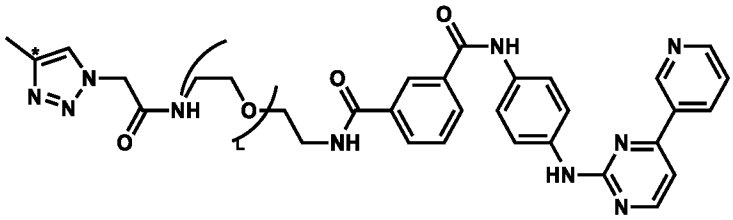
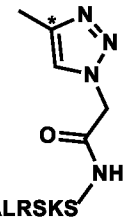
Formula (I-2)

R[2] is independently selected from -C(O)-O-C$_{1-3}$alkyl, -C(O)-O-C$_{2-3}$alkenyl, -C(O)-O-C$_{2-3}$ alkynyl, -C(O)-O-aryl, -C(O)-C$_{1-3}$alkyl, -C(O)-C$_{2-3}$alkenyl, -C(O)-C$_{2-3}$alkynyl, -C(O)-aryl, -C(O)-NH$_2$, -C(O)-O-CH$_2$-C≡C-phenyl, -C(O)O-CH$_2$-triazolyl-CH$_2$-C(O)NH$_2$; C$_{1-3}$alkyl, C$_{2-3}$alkenyl, C$_{2-3}$alkynyl, carbocycle selected from cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, heterocycle selected from azetidine, oxetane, pyrrolidine, tetrahydrofuran, piperidine, pyran, morpholine, oxazine, dioxane, phenyl, 5-6 membered heteroaryl, halo-C$_{1-3}$alkyl, phenyl -C$_{1-3}$alkyl, preferably -C(O)-O-

methyl, -C(O)-O-ethyl, -C(O)-O-phenyl, -C(O)-phenyl or -C(O)-O-CH_2-C≡CH, -C(O)-O-CH_2-C≡C-phenyl, C(O)O-CH_2-R^8

R^3 is independently selected from -C(O)-O-C_{1-3}alkyl, -C(O)-O-C_{2-3}alkenyl, -C(O)-O-C_{2-3} alkynyl, -C(O)-O-aryl, -C(O)-C_{1-3}alkyl, -C(O)-C_{2-3}alkenyl, -C(O)-C_{2-3}alkynyl, -C(O)-aryl, -C(O)-NH_2, -C(O)-O-CH_2-C≡C-phenyl, -C(O)O-CH_2-triazolyl-CH_2-C(O)NH_2; preferably -C(O)-O-methyl, -C(O)-O-ethyl, -C(O)-O-phenyl, -C(O)-phenyl or -C(O)-O-CH_2-C≡CH, -C(O)-O-CH_2-C≡C-phenyl, C(O)O-CH_2-R^8

wherein,

R^8 is  ,  ,  ,  ,  ,  LARRRALRSKS where * is the point of attachment and L = 1-3;

preferably, the compound of formula (I) is a compound of formula (I-1), and wherein one of R^2 and R^3 are as defined herein,
more preferably
R^3 is selected from -C(O)-O-C_{1-3}alkyl, -C(O)-O-C_{2-3}alkenyl, -C(O)-O-C_{2-3} alkynyl, -C(O)-O-aryl, -C(O)-C_{1-3}alkyl, -C(O)-C_{2-3}alkenyl, -C(O)-C_{2-3}alkynyl, -C(O)-aryl, -C(O)-NH_2, -C(O)-O-CH_2-C≡C-phenyl; C(O)O-CH_2-R^8
wherein R^8 is as defined in claims 1 or 2, or preferably any of claims 3 to 8,
preferably -C(O)-O-methyl, -C(O)-O-ethyl, -C(O)-O-phenyl, -C(O)-phenyl or -C(O)-O-CH_2-C≡CH, -C(O)-O-CH_2-C≡C-phenyl, and
R^2 is selected from methyl, cyclohexyl, phenyl, -CH_2-phenyl, -CH_2-CH_2-CH_2Cl, -CH_2-CH=CH_2, 4-Br-phenyl, or together with R^5 may form a -CH_2-C(CH_2)- group, and
R^6 is as defined in any of claims 1 to 9, preferably in claim 3 or claim 4; or

preferably, the compound of formula (I) is a compound of formula (I-2), and wherein one of R^2 and R^3 are as defined herein,
more preferably
R^3 is selected from -C(O)-O-C_{1-3}alkyl, -C(O)-O-C_{2-3}alkenyl, -C(O)-O-C_{2-3} alkynyl, -C(O)-O-aryl, -C(O)-C_{1-3}alkyl, -C(O)-C_{2-3}alkenyl, -C(O)-C_{2-3}alkynyl, -C(O)-aryl, -C(O)-NH_2, -C(O)-O-CH_2-C≡C-phenyl, -C(O)O-CH_2-triazolyl-CH_2-C(O)NH_2; -C(O)O-CH_2-R^8
wherein R^8 is as defined in claims 1 or 2, or preferably any of claims 3 to 8,
preferably -C(O)-O-methyl, -C(O)-O-ethyl, -C(O)-O-phenyl, -C(O)-phenyl or -C(O)-O-CH_2-C≡CH, -C(O)-O-CH_2-C≡C-phenyl, and
R^2 is selected from methyl, cyclohexyl, phenyl, -benzyl, -CH_2-CH_2-CH_2Cl, -CH_2-CH=CH_2, 4-Br-phenyl, or together with R^5 may form a -CH_2-C(CH_2)- group, and
R_6 is as defined in any of claims 1 to 8, preferably in claim 2 or claim 3.

10. Compound according to any of the previous claims or pharmaceutically acceptable salts thereof, wherein

R^6 is 3-7 membered heterocyclyl, C_{6-10} aryl, 5-7 membered heteroaryl, -C(O)O-C_{1-4} alkyl, -C(O)O-C_{2-4} alkenyl, -C(O)O-C_{2-4} alkynyl, -C(O)O-C_{6-10} aryl, -C(O)NH_2, -C(O)NHC_{1-4} alkyl, -C(O)NHC_{2-4} alkenyl, -C(O)NHC_{2-4} alkynyl, -C(O)NC_{1-4} $alkyl_2$, -C(O)NC_{2-4} $alkenyl_2$, -C(O)NC_{2-4} $alkynyl_2$, -C(O)NH-5-10 membered heteroaryl, -C(O)O-C_{1-4} alkyl-C≡CR^7, -C(O)O-C_{1-4}-alkyl-R^8

R^7 is H, C_{6-10} aryl, 5-7 membered heteroaryl, vinyl, -$[(CH_2)_2O]_n$-C(O)OH, -$[(CH_2)_2O]_n$-C(O)OC_{1-4} alkyl, -$[(CH_2)_2O]_n$-C(O)NH_2, -$[(CH_2)_2O]_n$-C(O)NHC_{1-4} alkyl, -$[(CH_2)_2O]_n$-C(O)NC_{1-4} $alkyl_2$-$[(CH_2)_2O]_n$-NH_2, -$[(CH_2)_2O]_n$-NHC_{1-4} alkyl, -$[(CH_2)_2O]_n$-NC_{1-4} $alkyl_2$, -C(O)O-$[(CH_2)_2O]_n$- C_{6-10} aryl, -C(O)O-$[(CH_2)_2O]_n$-5-7 membered heteroaryl, -

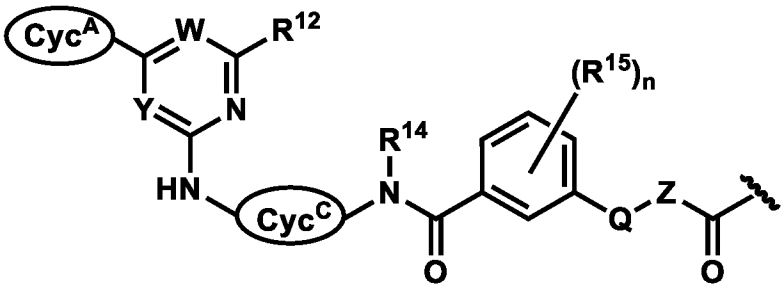
$C(O)NH(CH_2)_nNHC(O)$ C_{6-10} aryl, $-C(O)NH(CH_2)_nNHC(O)$-5-7 membered heteroaryl, preferably H, C_{6-10} aryl, 5-7 membered heteroaryl

R^8 is 5-6-membered heterocyclyl, the heterocyclyl is optionally substituted with one or more substituent independently selected from C_{1-4} alkyl, C_{2-4} alkenyl, C_{2-4} alkynyl, C_{3-6} carbocyclyl, 3-6 membered heterocyclyl, C_{6-8} aryl, 5-10 membered heteroaryl, the aryl and heteroaryl are optionally substituted with one or more substituent independently selected from C_{1-4} alkyl, halogen, $-NH_2$, $-NHC_{1-4}$ alkyl, $-N(C_{1-4}$ alkyl$)_2$;
halo-C_{1-4} alkyl, aryl-C_{1-4} alkyl, $-C(O)O-C_{1-4}$ alkyl, $-C(O)O-C_{2-4}$ alkenyl, $-C(O)O-C_{6-10}$ aryl, $-C(O)-C_{1-4}$ alkyl, $-C(O)-C_{2-4}$ alkenyl, $-C(O)-C_{2-4}$ alkynyl, $-C(O)-C_{6-10}$ aryl, $-C(O)NH_2$, $-C(O)NHC_{1-4}$ alkyl, $-C(O)NHC_{2-4}$ alkenyl, $-C(O)NHC_{2-4}$ alkynyl, $-C(O)NC_{1-4}$ alkyl$_2$, $-C(O)NC_{2-4}$ alkenyl$_2$, $-C(O)NC_{2-4}$ alkynyl$_2$, $-CN$, $-SO_3H$, $-SO_3C_{1-4}$ alkyl, $-CF_3$, $-C_{1-4}$ alkyl-$C(O)O-C_{1-4}$ alkyl, $-C_{1-4}$ alkyl-C(O)OH, $-C_{1-4}$ alkyl-$C(O)O-C_{2-4}$alkenyl, $-C_{1-4}$alkyl-$C(O)NH_2$, $-C_{1-4}$ alkyl-$C(O)NH_2$, $-C_{1-4}$ alkyl-$C(O)NHC_{1-4}$ alkyl, $-C_{1-4}$ alkyl-$C(O)NC_{1-4}$ alkyl$_2$, $-[(CH_2)_2O]_n-C(O)OH$, $-[(CH_2)_2O]_n-C(O)OC_{1-4}$ alkyl, $-[(CH_2)_2O]_n-NH_2$, $-[(CH_2)_2O]_n-NHC_{1-4}$ alkyl, $-[(CH_2)_2O]_n-NC_{1-4}$ alkyl$_2$, $-CH_2-C(O)NH(CH_2)_nNHC(O)-C_{6-10}$ aryl, $-CH_2-C(O)O(CH_2)_nNHC(O)-C_{6-10}$ aryl, $-CH_2-C(O)NH(CH_2)_nOC(O)-C_{6-10}$ aryl, $-CH_2-C(O)O(CH_2)_nOC(O)-C_{6-10}$ aryl, $C(O)NH(CH_2)_nNHC(O)$-5-7 membered heteroaryl wherein
n = 1-6.

11. Compound according to any of the previous claims or pharmaceutically acceptable salts thereof, wherein

R^6 is -C(O)O-methyl, -C(O)O-ethyl, -C(O)O-*tert*butyl, $-C(O)O-CH_2$-phenyl, -C(O)NH-*tert*butyl, -C(O)NH-phenyl, $-C(O)NH-CH_2$-phenyl, $-C(O)NH-C(Me)_2-CH_2-OH$, $-C(O)NH-CH_2-C(Me)_2-OH$, preferably -C(O)O-*tert*butyl, -C(O)Obenzyl, 5-methyloxazol-2-yl, quinolin-8-yl.

12. Compound according to any of claims 1-9 or pharmaceutically acceptable salts thereof, wherein

R^6 is 

wherein

Cyc^A is an optionally substituted aryl or heteroaryl ring, preferably an optionally substituted phenyl or pyridine ring, more preferably an optionally substituted 3-pyridinyl ring; even more preferably an unsubstituted 3-pyridinyl ring;

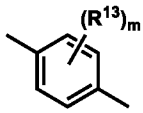
Y is N or CR^{10}, preferably N

W is CR^{11} or N, preferably CR^{11}

R^{10} is H or C_{1-6} alkyl, preferably H

R^{11} is H or C_{1-6} alkyl, preferably H

R^{12} is H, halogen, C_{1-6} alkyl, preferably H or Cl, more preferably H

Cyc^C is phenyl optionally substituted with R^{13} group(s) or a phenyl isostere, preferably 

or 

R^{13} is halogen, C_{1-6} alkyl, halo-C_{1-6} alkyl, preferably Cl, methyl or trifluoromethyl, more preferably methyl

m is an integer of 0 to 4, preferably 0 or 1

R^{14} is H, C_{1-6} alkyl or N-protecting group, preferably H, methyl, ethyl, or an N-protecting group, more preferably H

R^{15} is halogen, C_{1-6} alkyl, halo-C_{1-6} alkyl, preferably C_{1-3} alkyl, preferably halogen, methyl, ethyl, or trifluoromethyl, preferably H or methyl,

n is an integer of 0 to 4, preferably 0, 1 or 2, more preferably 0 or 1

Q is direct bond or $–[-CHR^{16}-]_o-$

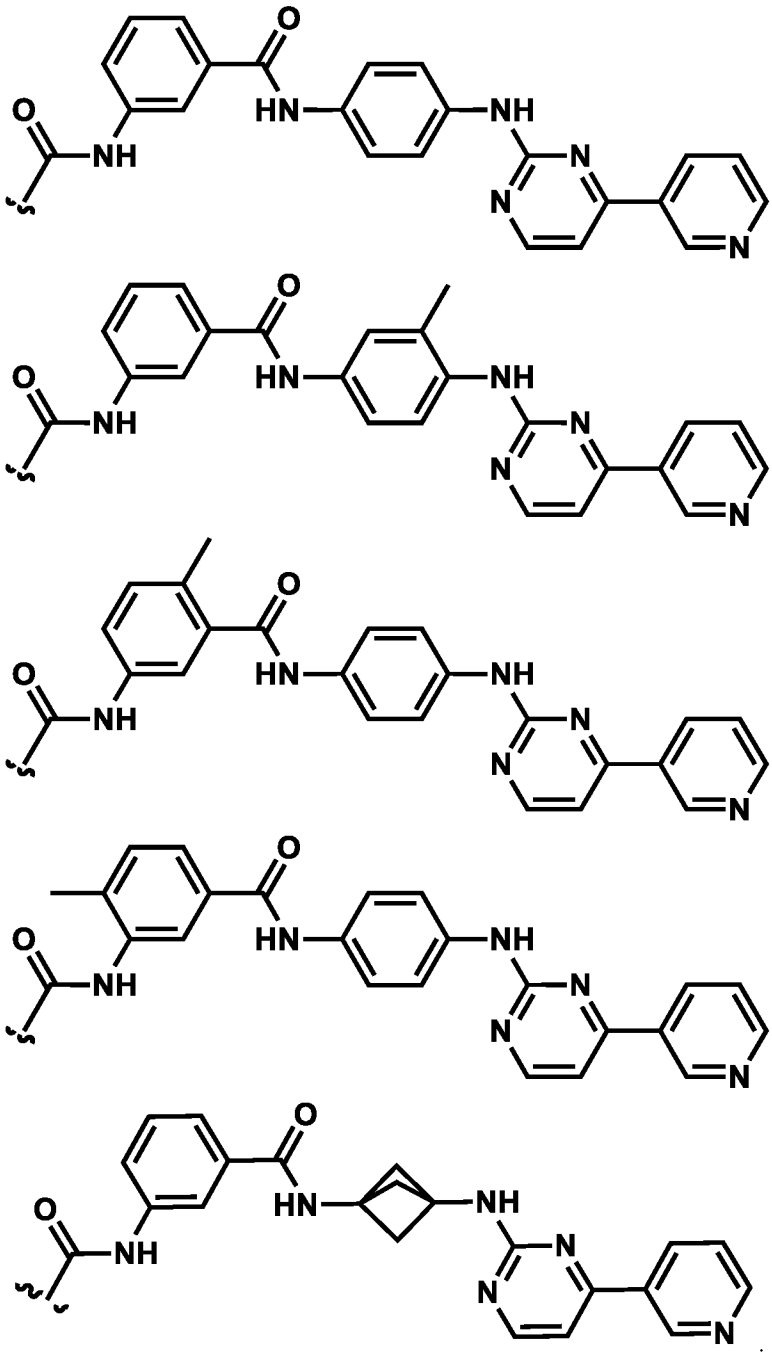
R^{16} is H or C_{1-6} alkyl, preferably H

o is an integer of 1 or 2, preferably 1

Z is NH or O

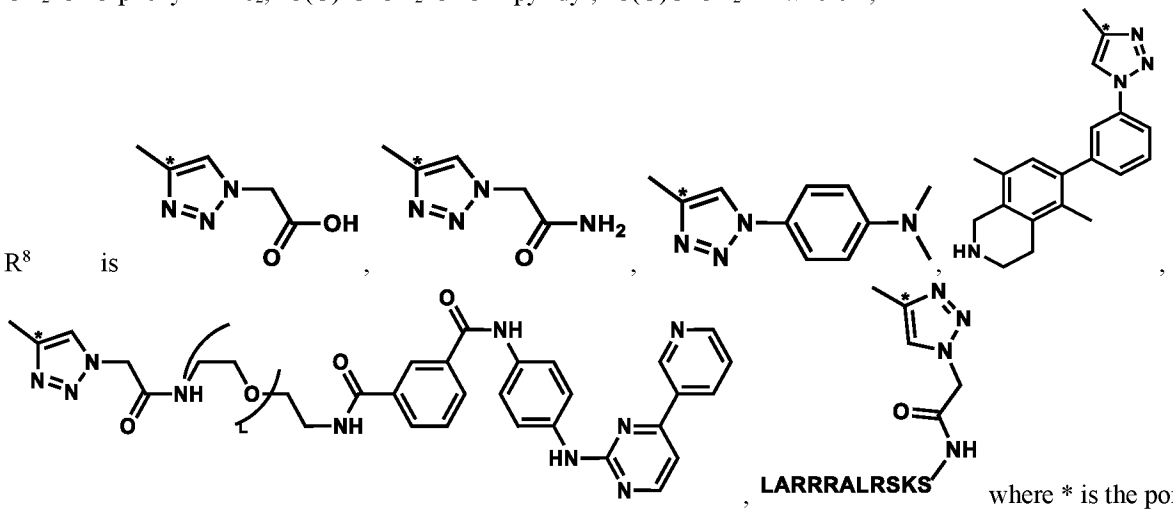
13. Compound according to claim 12 or pharmaceutically acceptable salts thereof, wherein

R^6 is selected from



14. Compound according to any of claims 1-10 or pharmaceutically acceptable salts thereof, wherein

R^3 is C(O)-O-C_{1-4}alkyl, C(O)-O-C_{2-3}alkynyl, C(O)-aryl; preferably methyl, C(O)-O-methyl, C(O)-O-ethyl, C(O)-phenyl or -C(O)-O-CH_2-C≡CH, -C(O)-O-$(CH_2)_2$-C≡CH, -C(O)-O-$(CH_2)_3$-C≡CH, -C(O)-O-$(CH_2)_4$-C≡CH, -C(O)-O-CH_2-C≡C-phenyl-NMe_2, -C(O)-O-CH_2-C≡C-4-pyridyl, -C(O)O-CH_2-R^8 wherein,

R^8 is 

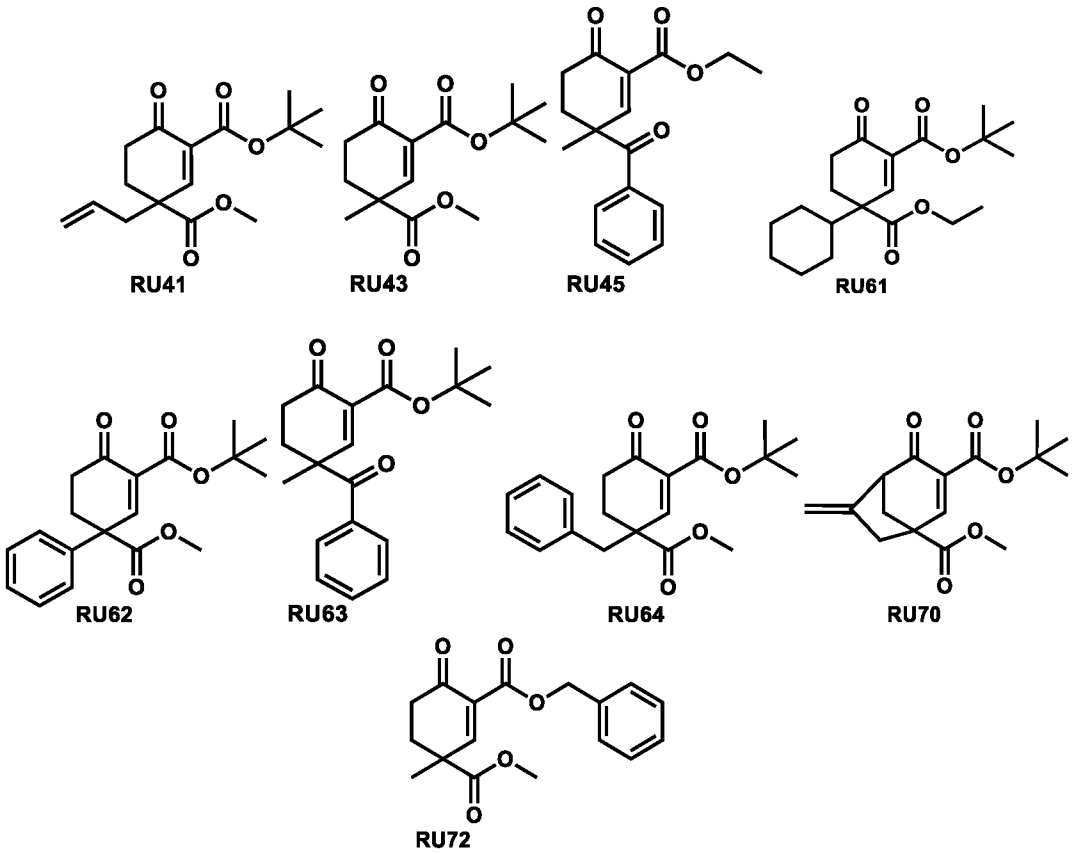
where * is the point of attachment and
L = 1-2

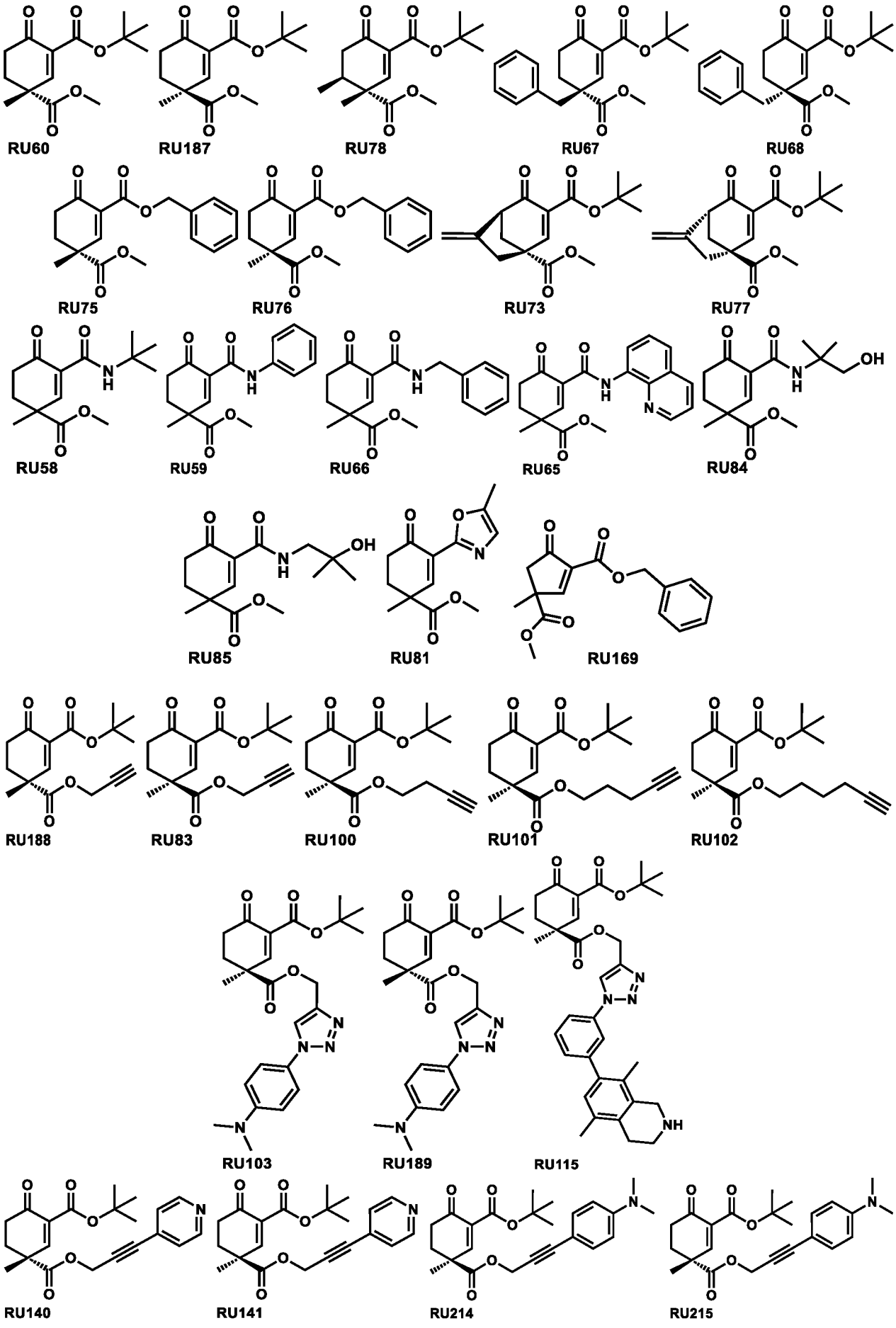
R^2 is C_{1-3}alkyl, C_{2-3}alkenyl, C_{2-3}alkynyl, cyclohexyl, halo-C_{1-3}alkyl, phenyl -C_{1-3}alkyl, preferably methyl, cyclohexyl, phenyl, benzyl, halo-C_{1-3}alkyl, -CH_2-CH_2-CH_2Cl, -CH_2-CH=CH_2,

or R^2 together with R^5 may form -CH_2-C(CH_2)-,

R^6 is -C(O)O^{t}Bu, -C(O)Obenzyl.

15. Compound according to claim 1 or pharmaceutically acceptable salts thereof, wherein the compound is selected from




RU60
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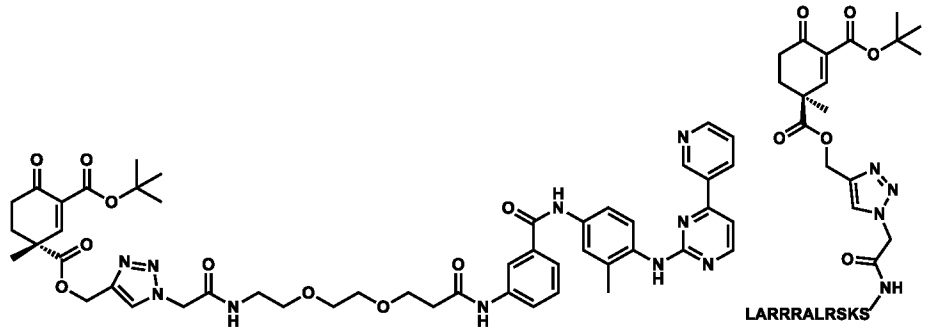
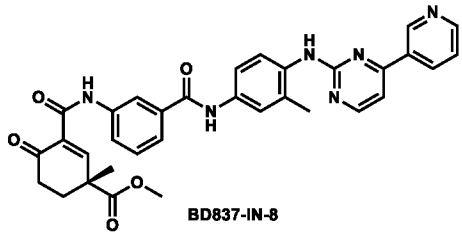


RU128 RU83-click-pepMNK1_C ;

and/or the compound is selected from



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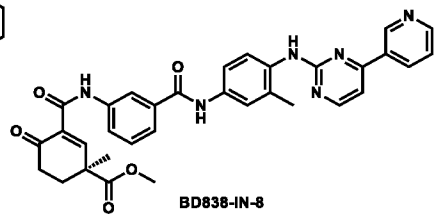

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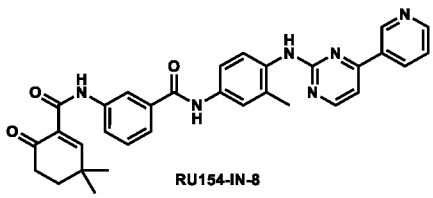

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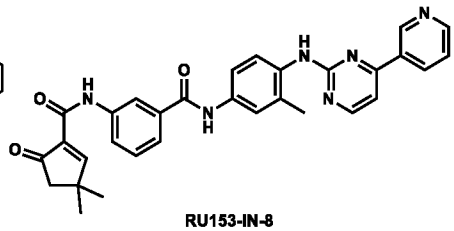

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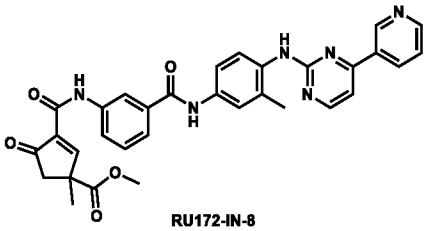

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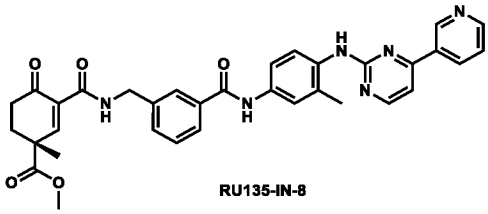

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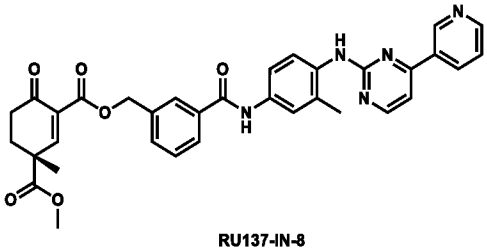

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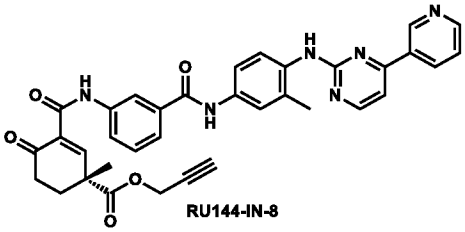

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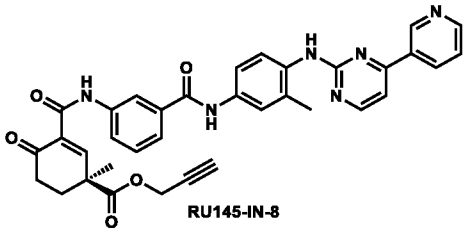

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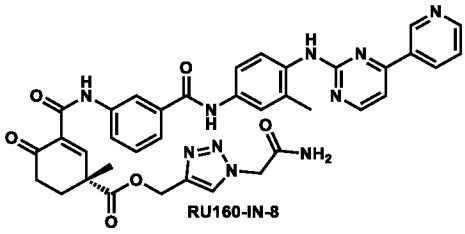

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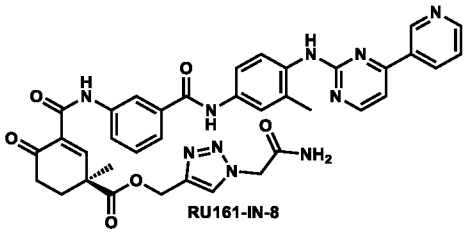
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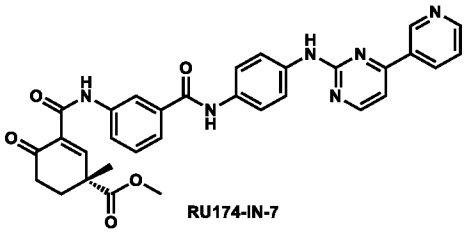
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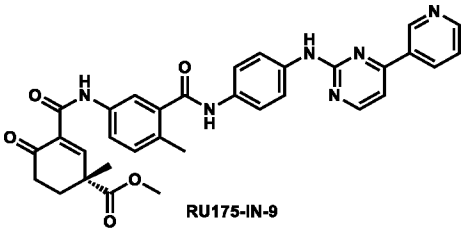
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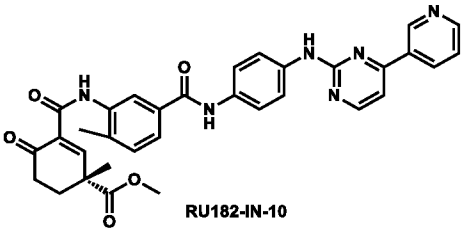
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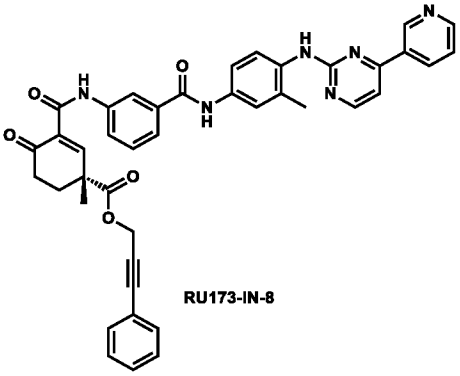
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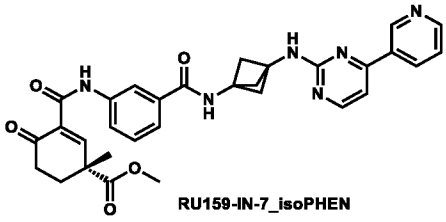
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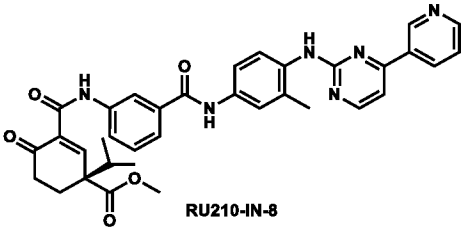
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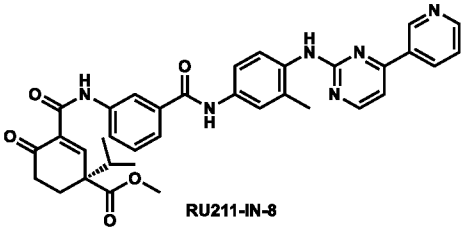
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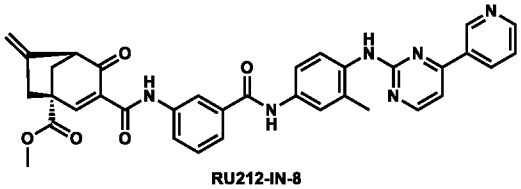
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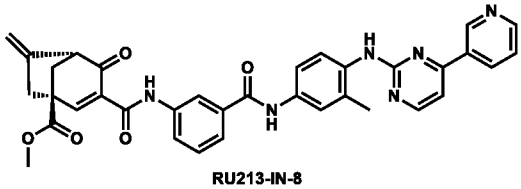
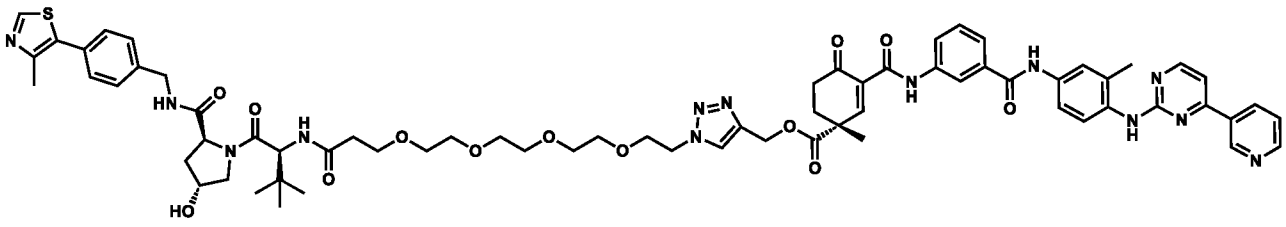

RU213-IN-8

and/or the compound is selected from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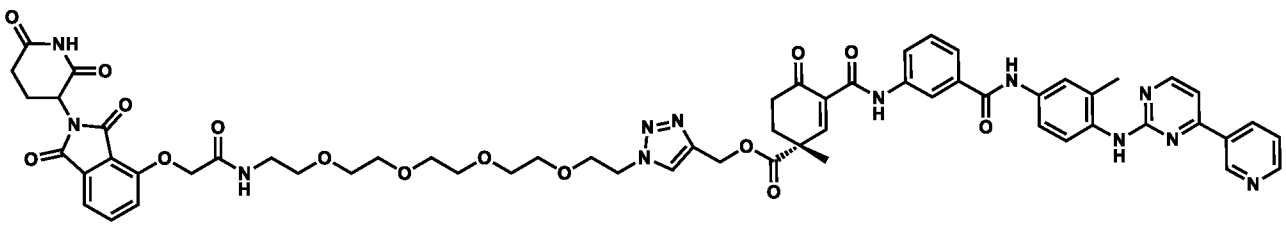
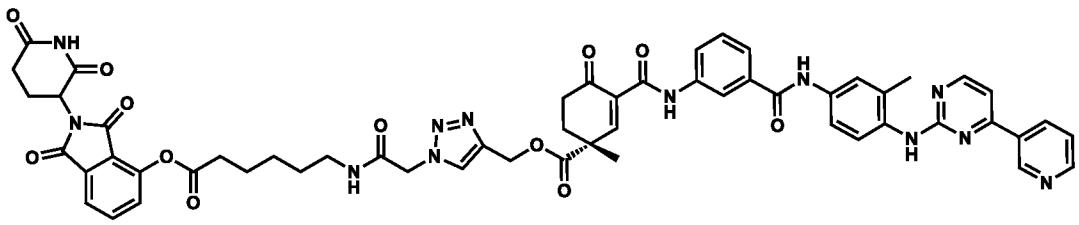
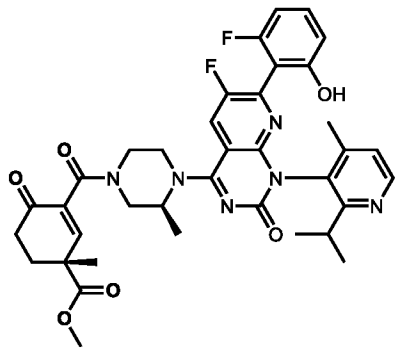

RU231



RU232

and/or the compound is



RU230.

16. The compound of any one of claims 1 to 15 which binds to a protein as a Michael-acceptor type protein binding compound or molecule, preferably said binding is covalent and reversible.

17. The compound of claim 16, wherein said binding is detectable by any assay suitable to detect protein binding of organic molecules, for example surface plasmon resonance (SPR), isothermal calorimetry (ITC), fluorescence polarization (FP) or NanoBiT assay.

18. The compound of claim 16 or 17 which is capable of binding in particular to a Cys and/or a His of a protein, as a nucleophile.

19. The compound of any of claim 16 to 18 which is a Michael-acceptor type protein inhibitor, wherein activity of a protein is inhibited by binding of the compound to said protein; in particular binding is targeted to a site of said protein which is essential to protein activity, whereas the warhead moiety of the compound of the invention covalently and reversibly binds to a nucleophile amino acid residue.

20. The compound of claim 19, wherein the site essential to protein activity is a binding surface, preferably the binding surface is a part of the protein surface to which another protein, e.g. a binding partner, preferably a protein substrate/activator/deactivator/scaffold protein or protein domain is capable of binding which is essential to the activity of said protein to which the Michael-acceptor compound binds.

21. The compound of claim 19, wherein the site essential to protein activity is an active site region, wherein a part of the compound interferes with the active site of the protein, preferably the active site region comprises an appropriate nucleophile residue, preferably a Cys and/or a His residue, to which the warhead moiety of the Michael-acceptor compound binds; as well as the active site.

22. The compound according to any of claims 19-22, wherein the inhibition is detectable by any assay suitable to detect protein activity or inhibition of activity of said protein, for example biochemical enzymatic assays in vitro or cell-based assays capable of detecting protein levels or activity in cells (e.g. by western blotting using specific antibodies).

23. The compound of any of claim 16 to 18 wherein the protein binding Michael-acceptor compound is a (part of a) bifunctional molecule, comprising a Michael-acceptor type protein binding moiety and a further protein binding moiety capable of binding to a further protein and preferably a linker between the Michael-acceptor type protein binding molecule or moiety and the further protein binding moiety, preferably wherein the bifunctional molecule is a homobifunctional molecule or the bifunctional molecule is a heterobifunctional molecule.

24. The compound of claim 23, wherein the further protein binding moiety capable of binding to a further protein is a Michael-acceptor type protein binding moiety.

25. The compound of claim 23 or 24, wherein the further protein is a ligase protein, preferably the further protein is a ubiquitin ligase capable of ubiquitination of said protein as a target protein, preferably the compound of the invention is a part of a proteolysis targeting chimera (PROTAC).

26. Use, preferably ex vivo use of a Michael-acceptor compound of any one of claims points 1 to 25 as a protein activity modulator, wherein the compound binds to a Cys and/or His residue of said protein with a reversible covalent bond.

27. Use according to claim 26, wherein the binding surface is a part of the protein surface to which another protein, e.g. a binding partner can bind, in particular the binding partner may be a protein inhibitor whereby the Michael-acceptor compound, by blocking the binding of the inhibitor, serves as an activator of said protein, or in particular the binding partner is a protein agonist of said protein and the Michael-acceptor compound, by blocking the binding of the agonist, serves as an inhibitor of said protein.

28. Use, preferably ex vivo use of a compound of any one of points 1 to 25 as a Michael-acceptor type protein inhibitor, preferably binding of the Michael-acceptor type protein inhibitor is targeted to a site of said protein which is essential to protein activity, whereas the warhead moiety of the compound of the invention covalently and reversibly binds to a nucleophile amino acid residue, in particular the nucleophile amino acid is a Cys and/or a His residue.

29. The use of claim 28, wherein the site essential to protein activity is a binding surface, preferably the binding surface is as defined in the previous claims.

30. The use of claim 28 or 29, wherein the inhibition is detectable by any assay suitable to detect protein activity or inhibition of activity of said protein.

31. The ex vivo use of any of claims 28 to 30, wherein said compound of any one of claims 1 to 26 is used as a kinase inhibitor, preferably

the kinase inhibitor is an inhibitor of a kinase of the family of mitogen-activated protein kinases (MAP kinases or MAPK) or

the kinase inhibitor is an inhibitor of a kinase of the MAPK subfamily of extracellular regulated kinases 1 (ERK1) or

the kinase inhibitor is an inhibitor of a kinase of the MAPK subfamily of extracellular regulated kinases 2 (ERK2) or

the kinase inhibitor is an inhibitor of a kinase of the MAPK subfamily of c-Jun N-terminal kinases (JNK) or

the kinase inhibitor is an inhibitor of a kinase of the MAPK subfamily of p38 kinases or

the kinase inhibitor is an inhibitor of a kinase of the MAPK subfamily of extracellular regulated kinases 5 (ERK5),

preferably the kinase inhibitor is selected from the following kinases: ERK1/2, JNK, p38, more preferably ERK2, JNK1, JNK2, JNK3, p38alpha.

32. The ex vivo use of claim 31, wherein the compound binds to the MAPK D-groove, wherein preferably the compound is as defined in claims 16 or 31.

33. The ex vivo use of claim 31 or 32, wherein

the compound binds to a Cys residue of said protein with a reversible covalent bond, preferably the compound is RU60, RU67, RU68, RU75, RU76, RU128, RU81, RU169, RU188, RU83, RU100, RU101, RU102, RU103, RU189, RU115, RU140, RU141, RU214, RU215, or

the compound binds to a His residue of said protein with a reversible covalent bond, preferably the compound is RU77, or

the compound binds to either to a Cys or a His residue of said protein with a reversible covalent bond, preferably the compound is RU187.

34. The compound of any of claims 1 to 26 for use in the treatment of a disease in a subject.

35. The compound of any of claims 1 to 26 for use in modulating a drug target protein associated with a disease, preferably the Michael-acceptor compound of the invention is used as an inhibitor of a drug target protein associated with said disease.

36. The compound for use according to claim 34 or 35, wherein the Michael-acceptor compound of the invention is used as a kinase inhibitor.

37. The compound for use according to claims 34 to 36 for use in the treatment of a subject in need of inhibition of a kinase activated pathway, preferably the kinase activated pathway is a MAPK kinase activated pathway, wherein preferably the disease is selected from the group consisting of an autoimmune disease, an inflammatory disease, a cardiovascular disease, a neurological disease or a disease of the nervous system, or a neoplasm or a cancer or malignancy,

preferably an inflammatory disease, a neurological disease or a neoplasm or any variant thereof.

38. The compound for use according to claims 34 to 37, wherein the kinase activated pathway is selected from

an ERK1 kinase activated pathway,

an ERK2 kinase activated pathway,

an ERK5 kinase activated pathway,

a JNK1 kinase activated pathway,

a JNK2 kinase activated pathway,

a JNK3 kinase activated pathway,

a p38alpha kinase activated pathway,

a p38beta kinase activated pathway,

a p38gamma kinase activated pathway,

p38delta kinase activated pathway.

Preferably the kinase activated pathway is selected from

an ERK1 kinase activated pathway,

an ERK2 kinase activated pathway,

a JNK kinase activated pathway,

a p38 kinase activated pathway or

an ERK5 kinase activated pathway,

in particular the kinase activated pathway is an ERK1/2, JNK and/or p38 kinase activated pathway, more preferably ERK2, JNK1, JNK2, JNK3, p38alpha kinase activated pathway.

39. The compound for use according to any of claims 34 to 38, wherein said disease is selected from the group consisting of the following diseases:

- type 2 diabetes mellitus and obesity associated with insulin resistance

- a neoplasm or a cancer or malignancy, preferably a tumor, in particular a solid tumor, for example a breast, lung or colon tumor,

- a neurological disease or a disease of the nervous system, preferably selected from the group consisting of

neurological injuries, like acute brain injury, such as ischemic stroke (IS), intracerebral hemorrhage (ICH), subarachnoid hemorrhage (SAH), traumatic brain injury (TBI), spinal cord injury (SCI), epilepsy;

neurodegenerative diseases, such as Huntington's disease (HD), Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS);

neurodevelopment diseases, such as autism spectrum disorder (ASD) and cerebral palsy, as well as others;

infectious neurological diseases, such as meningitis and encephalitis; and

neurological tumors, such as neuroblastoma (NB), glioblastoma (GBM), glioma,.

- an inflammatory disease, preferably atopic dermatitis, arthritis, e.g. psoriatic arthritis, rheumatoid arthritis, Crohn disease, ulcerative colitis, atherosclerosis, multiple sclerosis (MS), chronic obstructive pulmonary disease (COPD) and asthma.

40. The compound for use according to any of claims 34 to 39, wherein said disease is a disease related to impaired cell cycle, in particular selected from neoplasm and a neurological disease which is a cell cycle impairment disease or an aberrant cell cycle disease.

41. The compound for use according to any of claims 34 to 40, wherein the kinase inhibitor is an inhibitor of a kinase of the family of mitogen-activated protein kinases (MAP kinases or MAPK), preferably the kinase is selected from extracellular regulated kinases 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), p38 kinase, and extracellular regulated kinase 5 (ERK5), preferably from ERK2, JNK1, p38.

42. The use of any of claims 26 to 33 and the compound for use according to any of claims 34 to 41, wherein the following disclaimers does not apply:

the disclaimer defined in point 1.1.1,

the disclaimer defined in point 1.1.2,

the disclaimer defined in point 1.1.3,

the disclaimer defined in point 1.1.4,

the disclaimer defined in point 1.1.5,

the disclaimer defined in point 1.1.6,

the disclaimer defined in point 1.1.7.

43. Pharmaceutical composition comprising the compound defined in any of claims 1 to 15 and a pharmaceutically acceptable excipient or carrier.

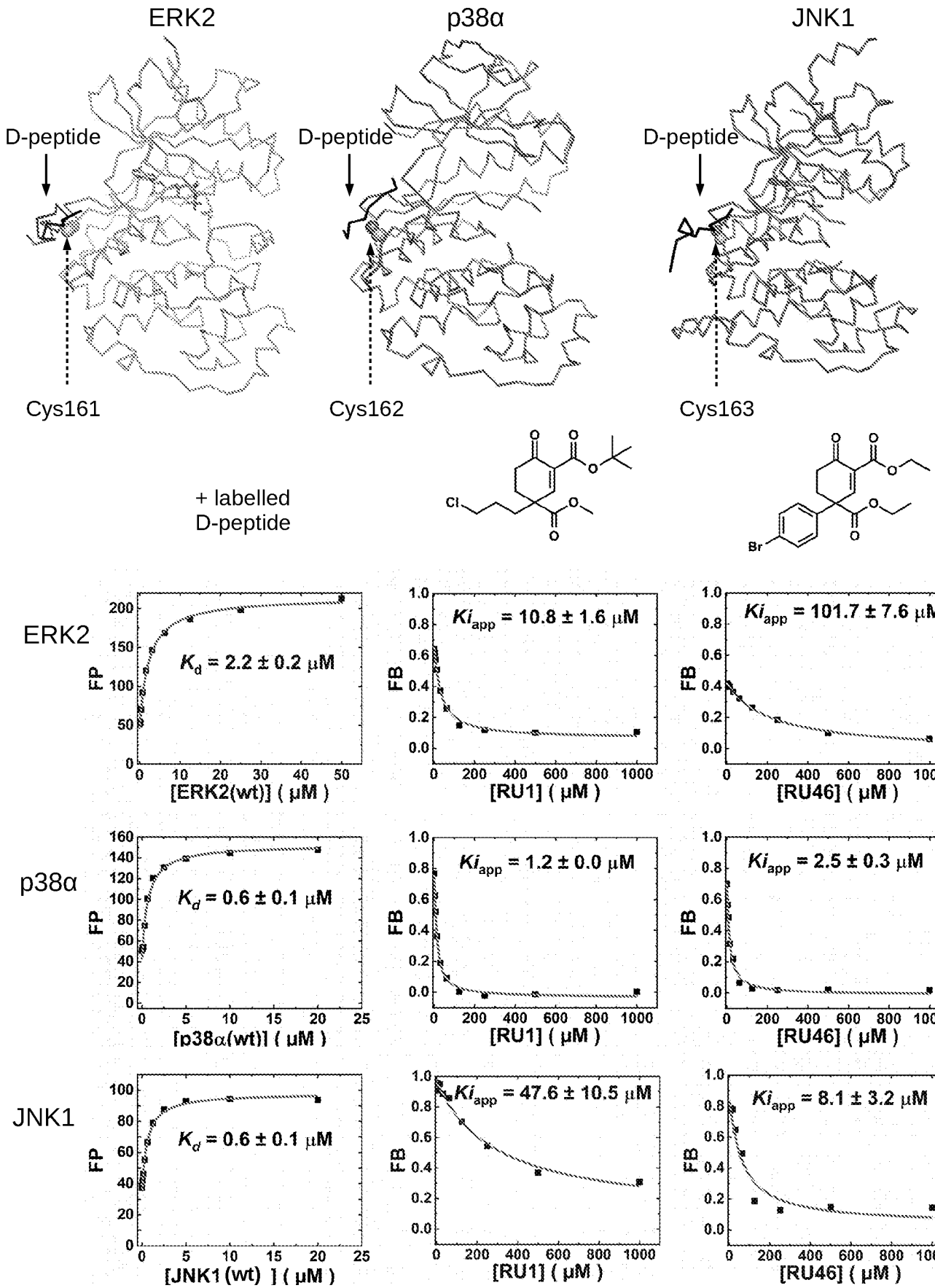
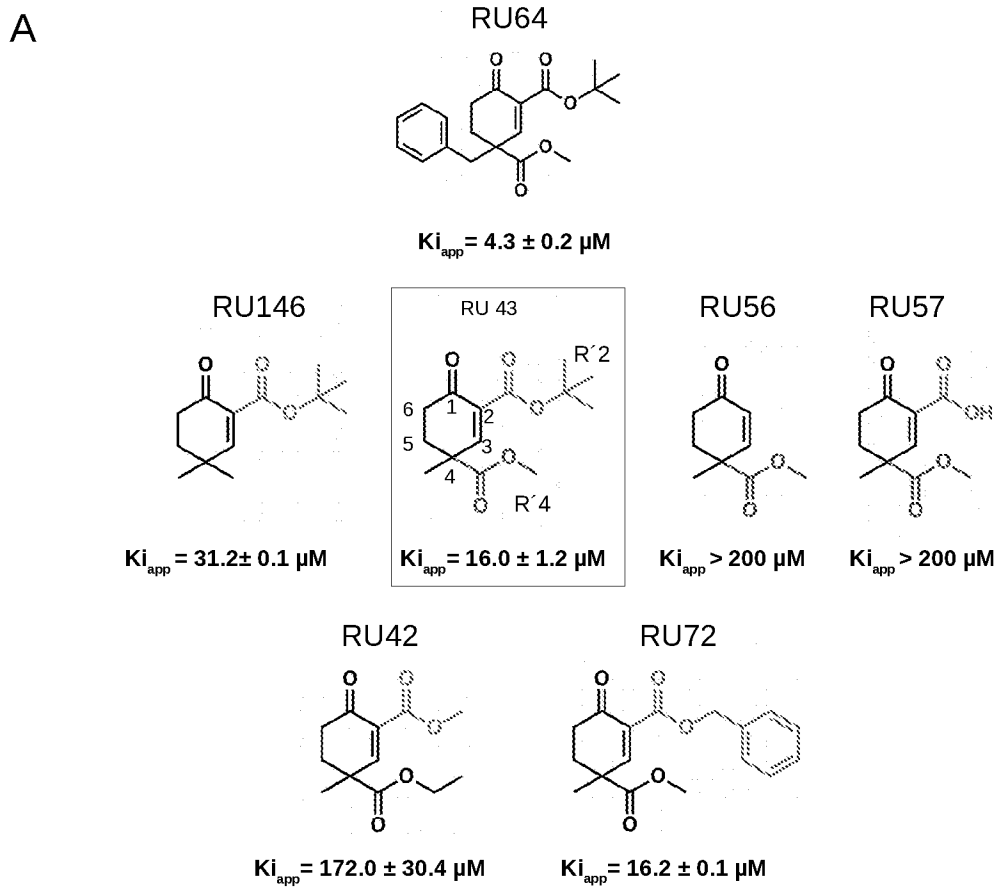

ERK2
p38α
JNK1
D-peptide
D-peptide
D-peptide
Cys161
Cys162
Cys163
+ labelled
D-peptide
Cl
Br
ERK2
p38α
JNK1
FP
FB
K_d = 2.2 ± 0.2 μM
K_d = 0.6 ± 0.1 μM
K_d = 0.6 ± 0.1 μM
Ki_{app} = 10.8 ± 1.6 μM
Ki_{app} = 101.7 ± 7.6 μM
Ki_{app} = 1.2 ± 0.0 μM
Ki_{app} = 2.5 ± 0.3 μM
Ki_{app} = 47.6 ± 10.5 μM
Ki_{app} = 8.1 ± 3.2 μM
[ERK2(wt)] (μM)
[p38α(wt)] (μM)
[JNK1 (wt)] (μM)
[RU1] (μM)
[RU46] (μM)

FIGURE 1


A
RU64
Ki app = 4.3 ± 0.2 µM
RU146
Ki app = 31.2± 0.1 µM
RU 43
R´2
R´4
Ki app = 16.0 ± 1.2 µM
RU56
Ki app > 200 µM
RU57
Ki app > 200 µM
RU42
Ki app = 172.0 ± 30.4 µM
RU72
Ki app = 16.2 ± 0.1 µM

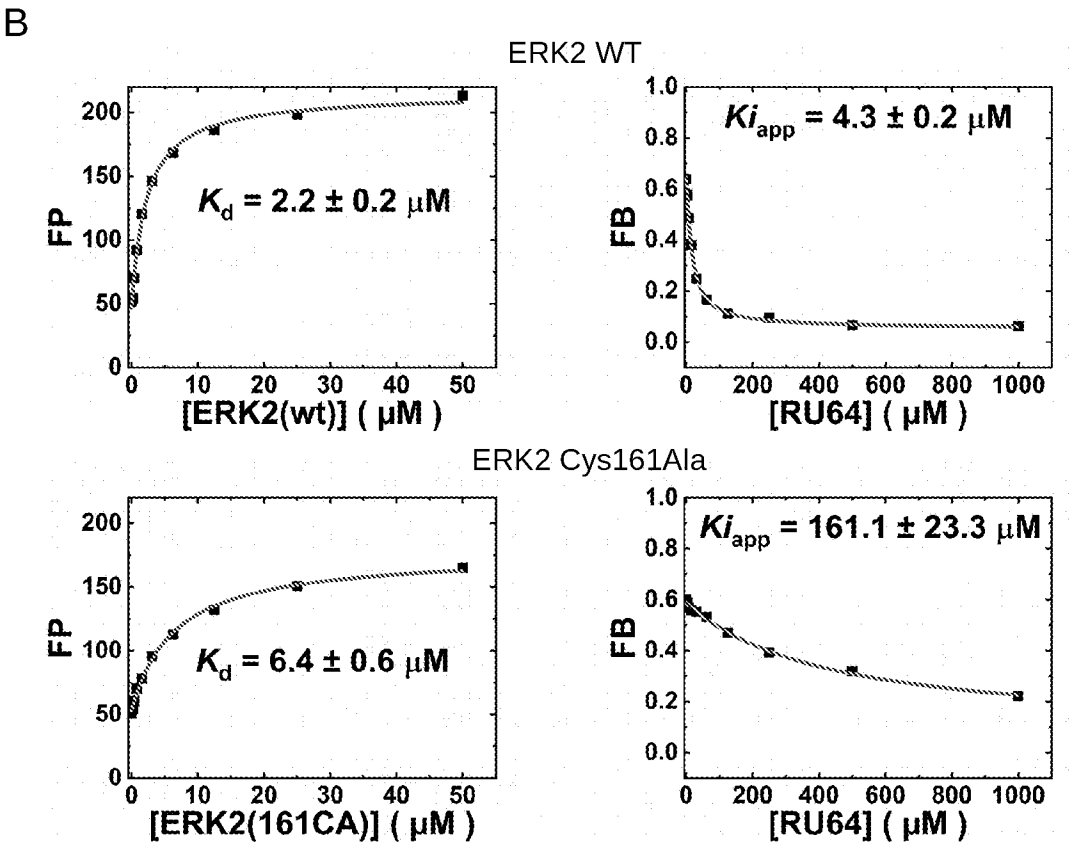

B
ERK2 WT
FP
Kd = 2.2 ± 0.2 µM
[ERK2(wt)] (µM)
FB
Kiapp = 4.3 ± 0.2 µM
[RU64] (µM)
ERK2 Cys161Ala
FP
Kd = 6.4 ± 0.6 µM
[ERK2(161CA)] (µM)
FB
Kiapp = 161.1 ± 23.3 µM
[RU64] (µM)

FIGURE 2

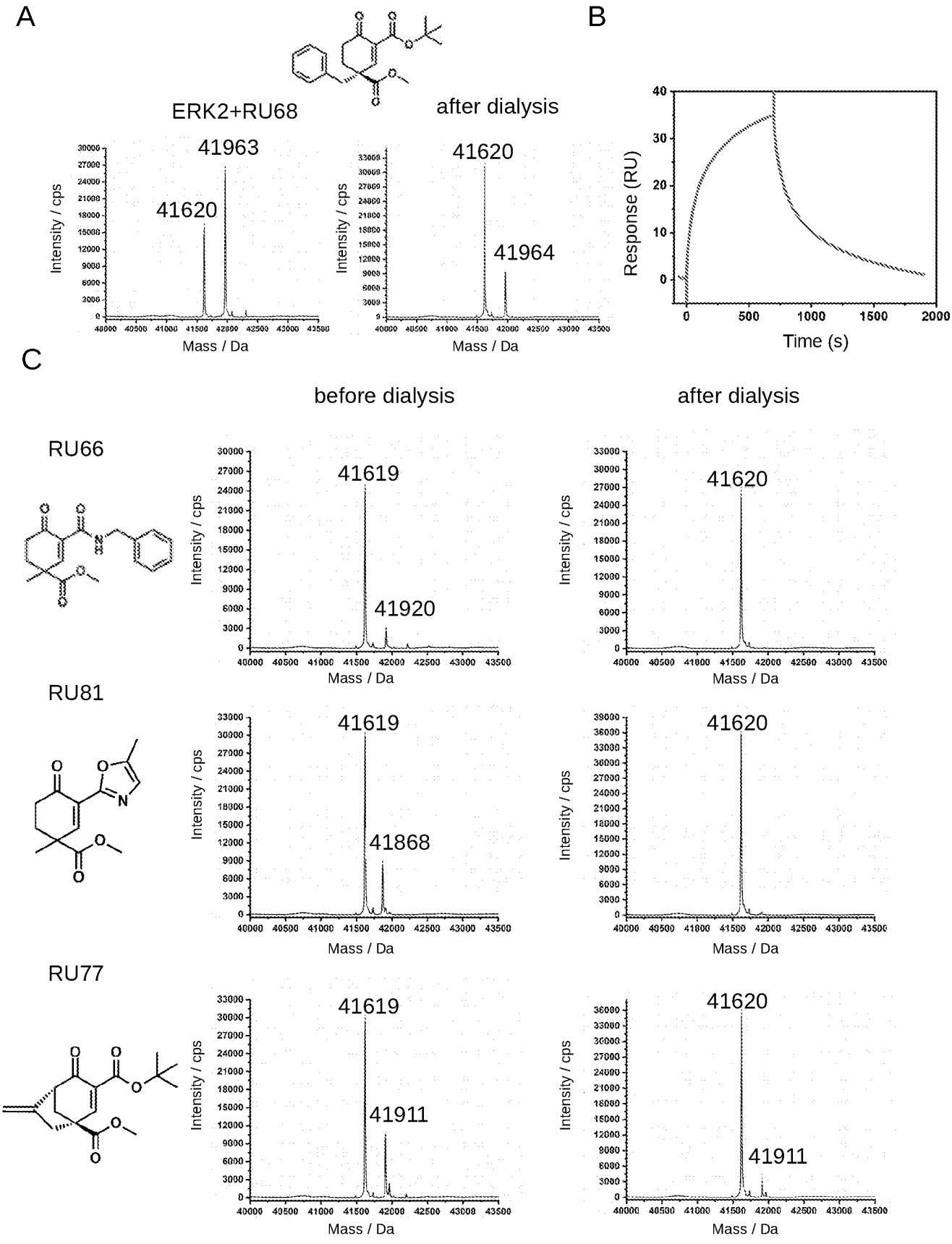
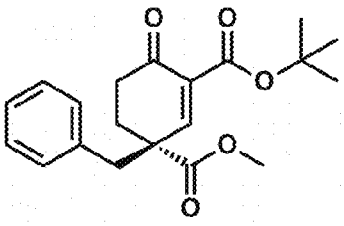
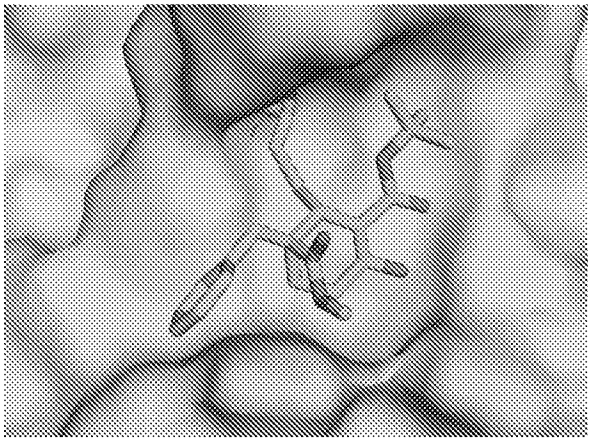

A
ERK2+RU68
41963
41620
Intensity / cps
Mass / Da
after dialysis
41620
41964
Intensity / cps
Mass / Da
B
Response (RU)
Time (s)
C
before dialysis
after dialysis
RU66
41619
41920
41620
RU81
41619
41868
41620
RU77
41619
41911
41620
41911

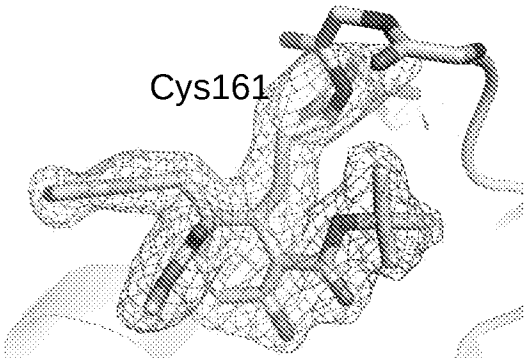
FIGURE 3

RU67





ERK2-RU67



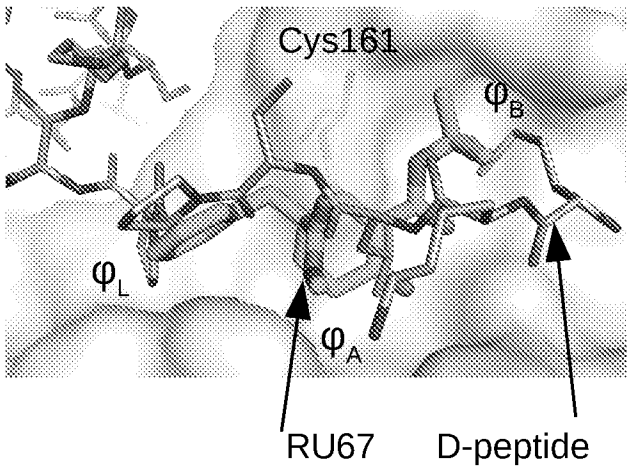
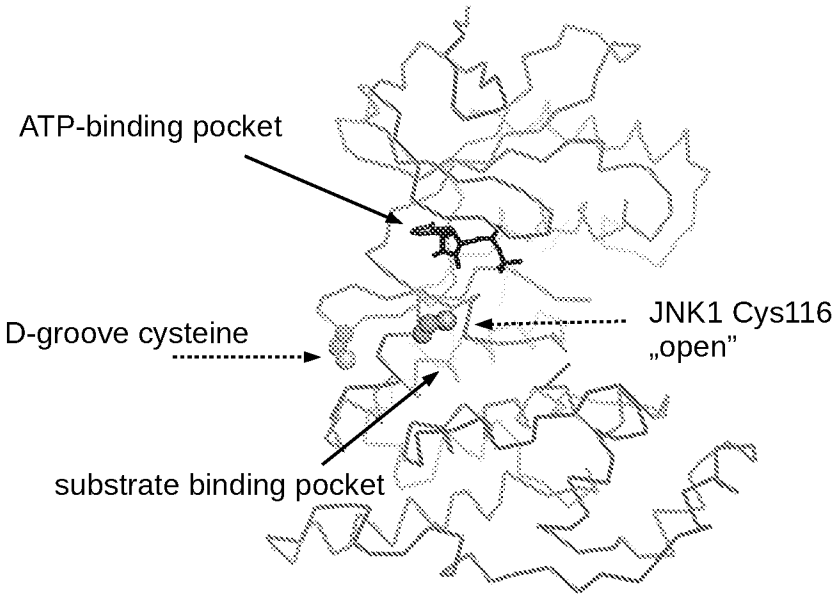
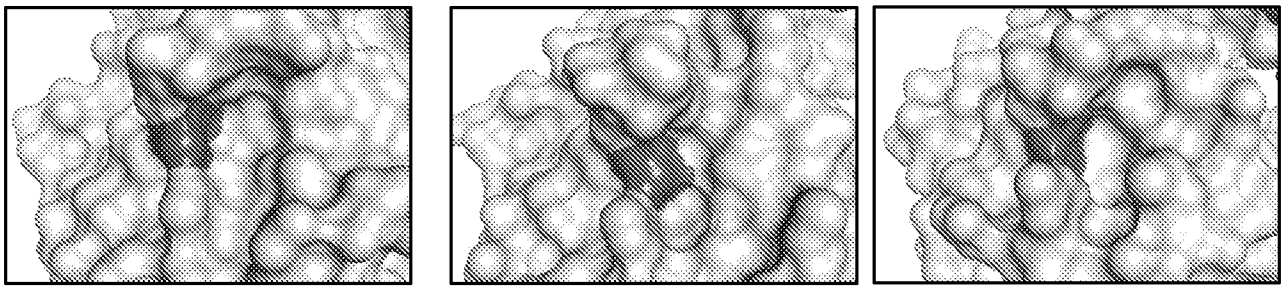


FIGURE 4

A



Cysteines in the **MAPK D-groove**: „saddle” position



ERK2, Cys161

p38α, Cys162

JNK3, Cys201
(Cys163 in JNK1)

Cys116 in JNK1: „open” position

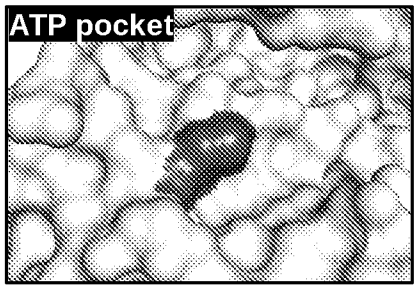
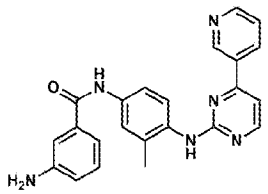


FIGURE 5-1

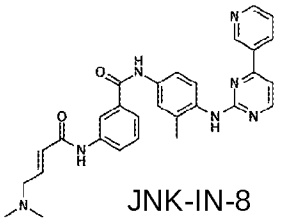
B

Directing scaffold (noncovalent)

IN-8 scaffold

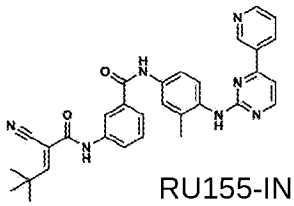


JNK1 (C116)



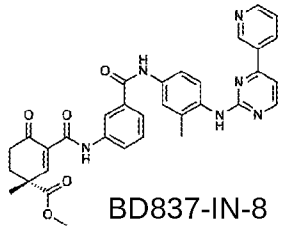
JNK-IN-8

K_i= 12 nM



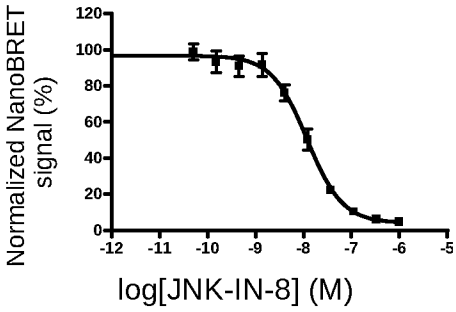
RU155-IN-8

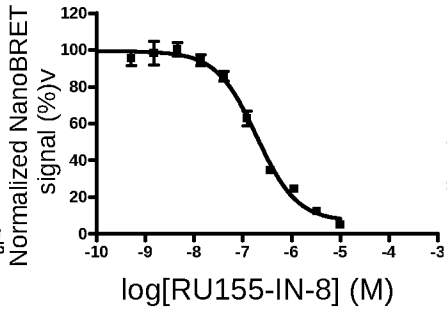
K_i= 192 nM

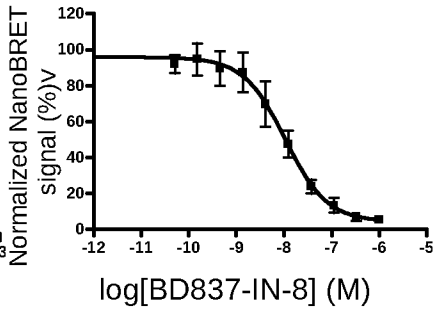


BD837-IN-8

K_i= 11 nM

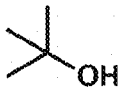




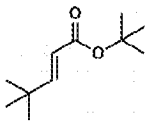


Directing group (noncovalent)

tert-butanol

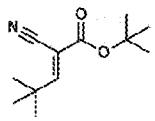


ERK2 (C161)



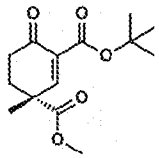
RU190

K_i > 200 µM



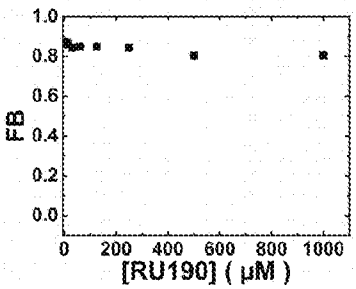
RU150

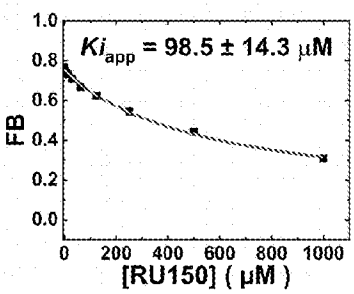
K_i = 98 µM



RU60

K_i = 12 µM





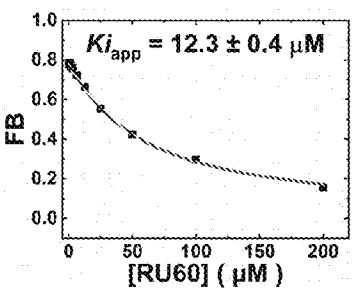
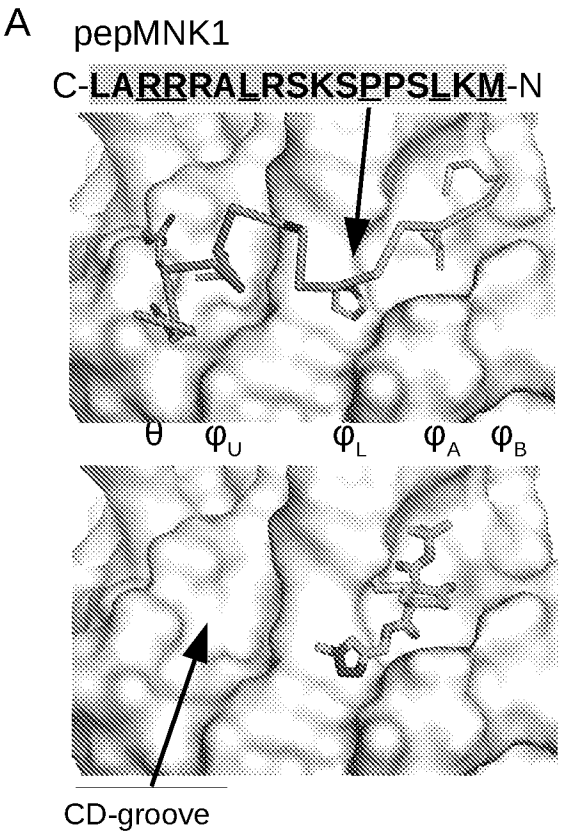
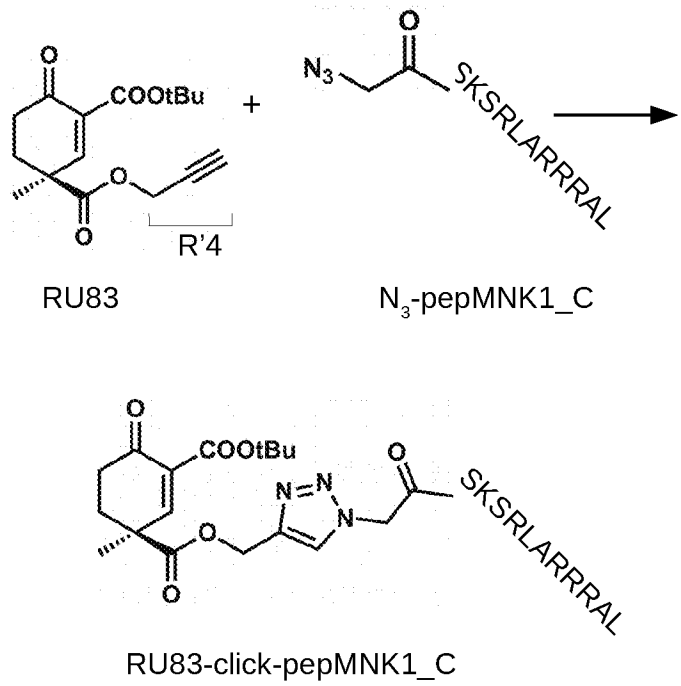


FIGURE 5-2

A

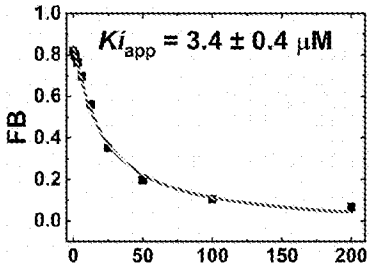


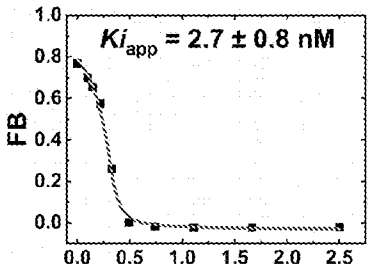
B



C

Name \ Ki (μM)	ERK2	p38α	JNK1
RU83	2.0	**3.4**	3.5
N_3-pepMNK1_C	32	>10	>200
RU83-click-pepMNK1_C	0.4	**0.0027**	0.8
pepMNK1	0.6	0.2	>200





D

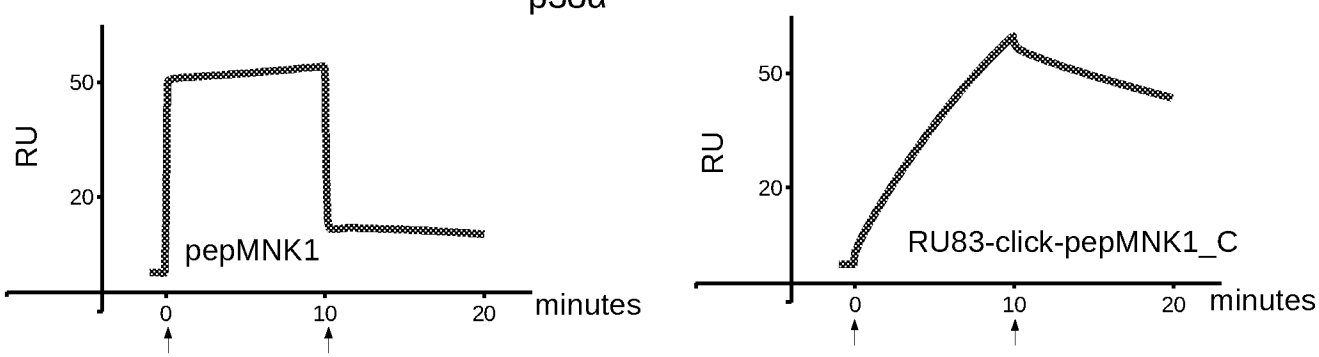
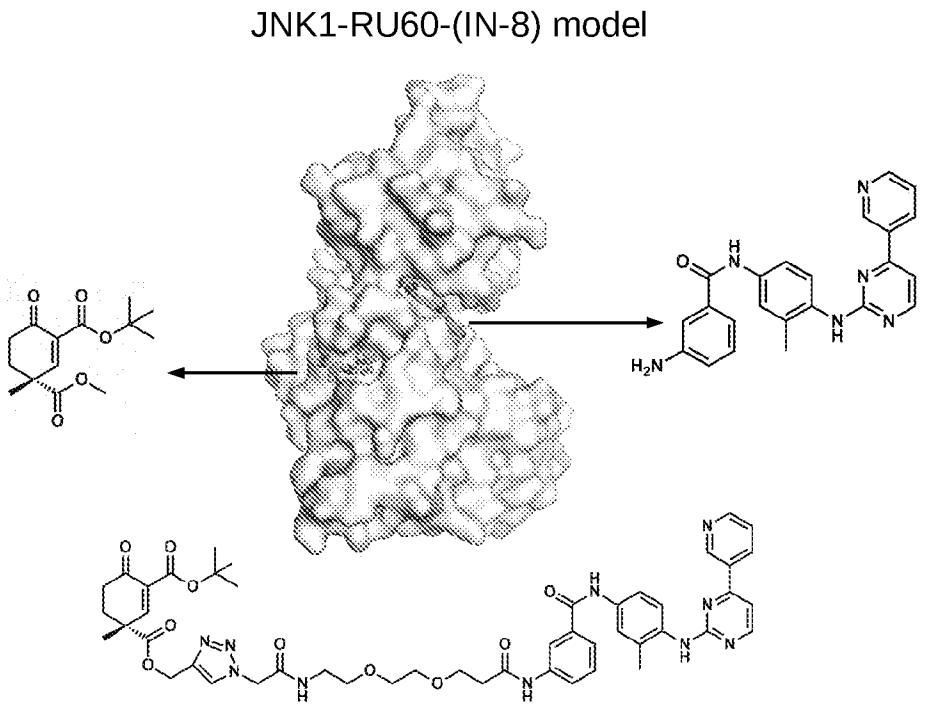


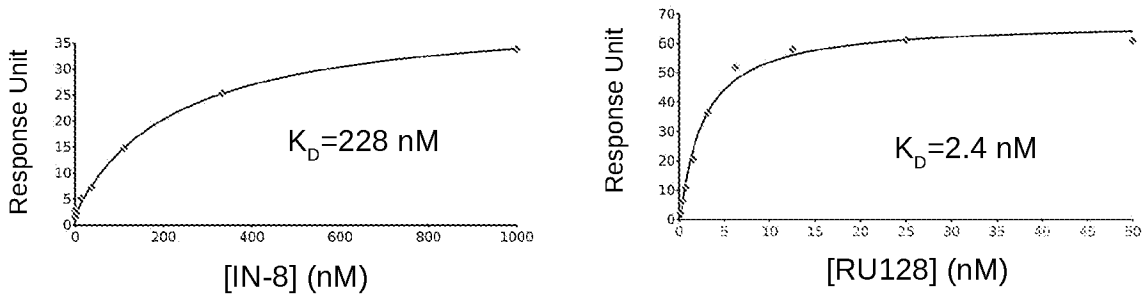
FIGURE 6

A



RU128: RU188-PEG-IN-8

B



C

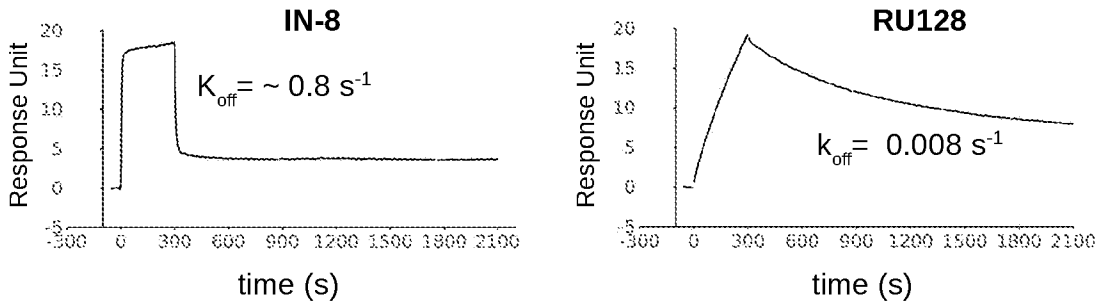
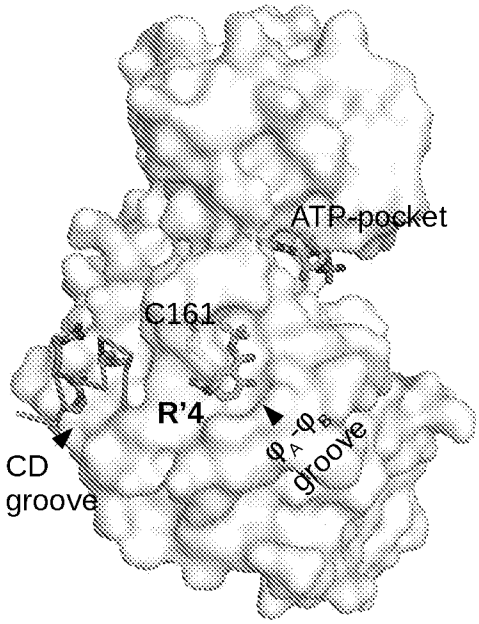
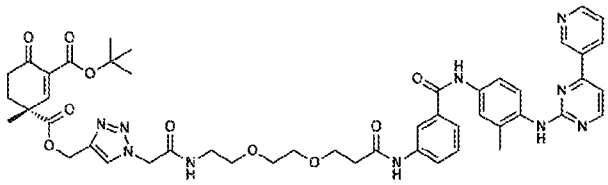


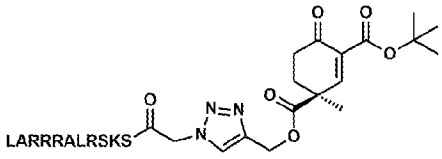
FIGURE 7

A



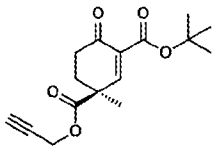


RU128: RU188-PEG-IN8

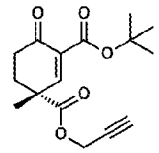


RU83-click-pepMNK1_C

B



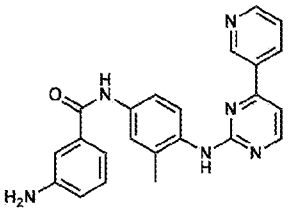
RU83



RU188

N_3-SKSRLARRRAL

N_3-pepMNK1_C

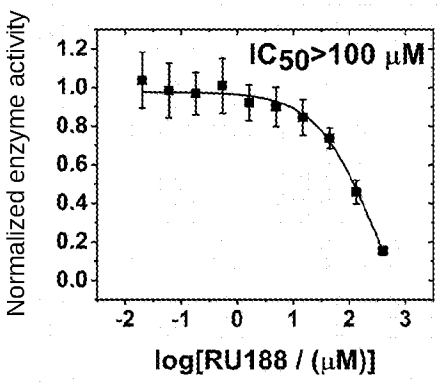


IN-8

compound	IC_{50} of D-SENSOR phosphorylation with 10 mM GSH (μM) for ERK2	p38α	JNK1
RU83	40.3	16.0	58.4
RU188	>100	72.4	**>100**
N_3-pepMNK1	>100	>100	>100
IN-8	>100	131.6	3.8
RU83-click-pepMNK1_C	8.7	0.4	15.6
RU128: RU188-PEG-IN-8	>100	2.4	**0.7**

CD groove

ATP pocket



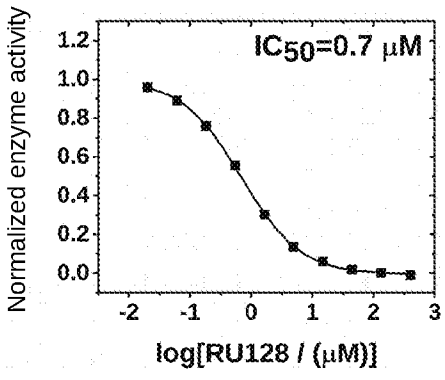


FIGURE 8

A

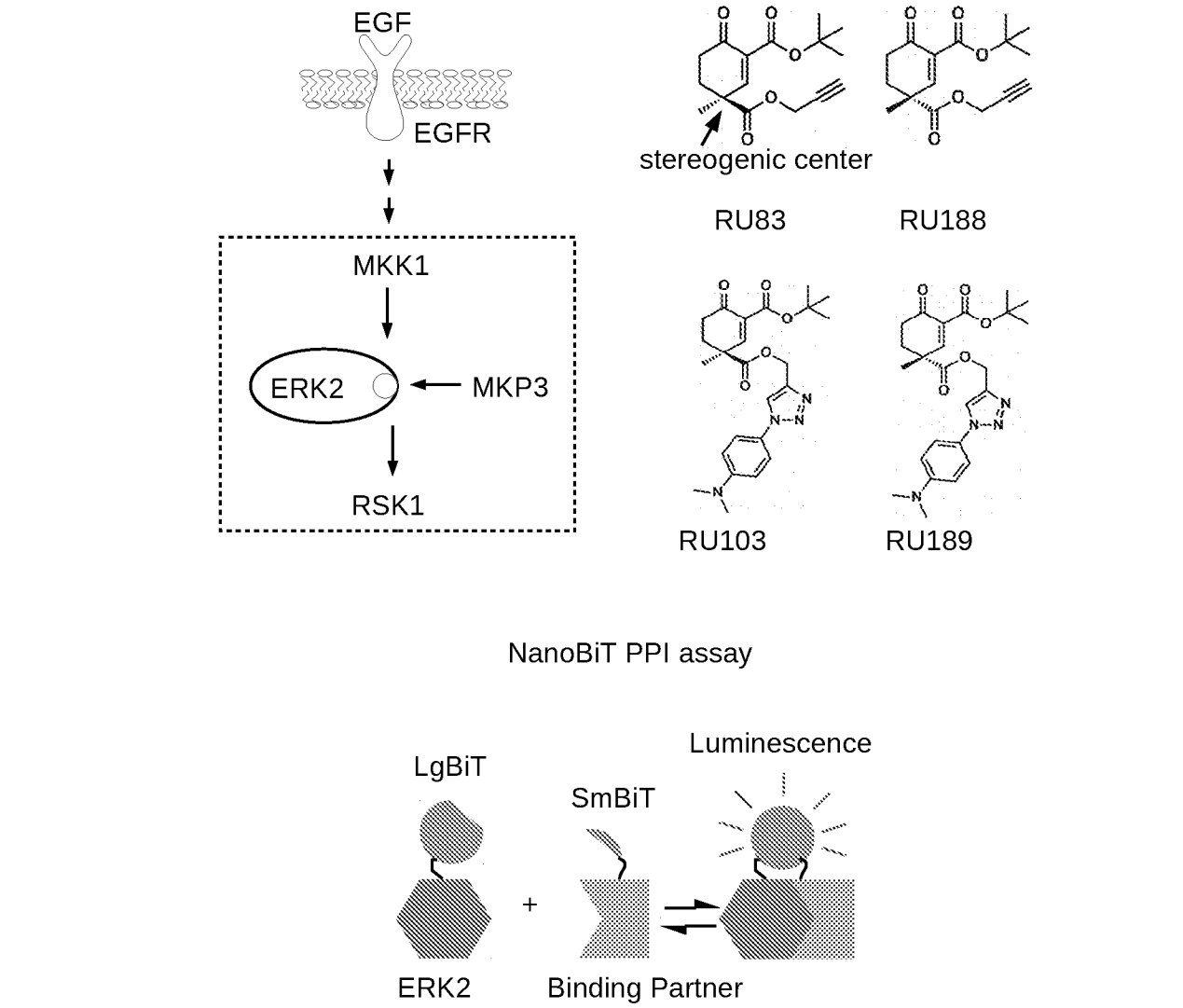

EGF
EGFR
MKK1
ERK2
MKP3
RSK1
stereogenic center
RU83
RU188
RU103
RU189
NanoBiT PPI assay
LgBiT
SmBiT
Luminescence
+
ERK2
Binding Partner
MKK1-ERK2
Luminescence (a.u.)
MKK1_D0
- + RU103 - + RU103
MKK1-ERK2
***
***
- + RU103 + RU189
ERK2-RSK1
NS
***
- + RU103 + RU189
ERK2-MKP3
***
***
- + RU103 + RU189

FIGURE 9-1

B

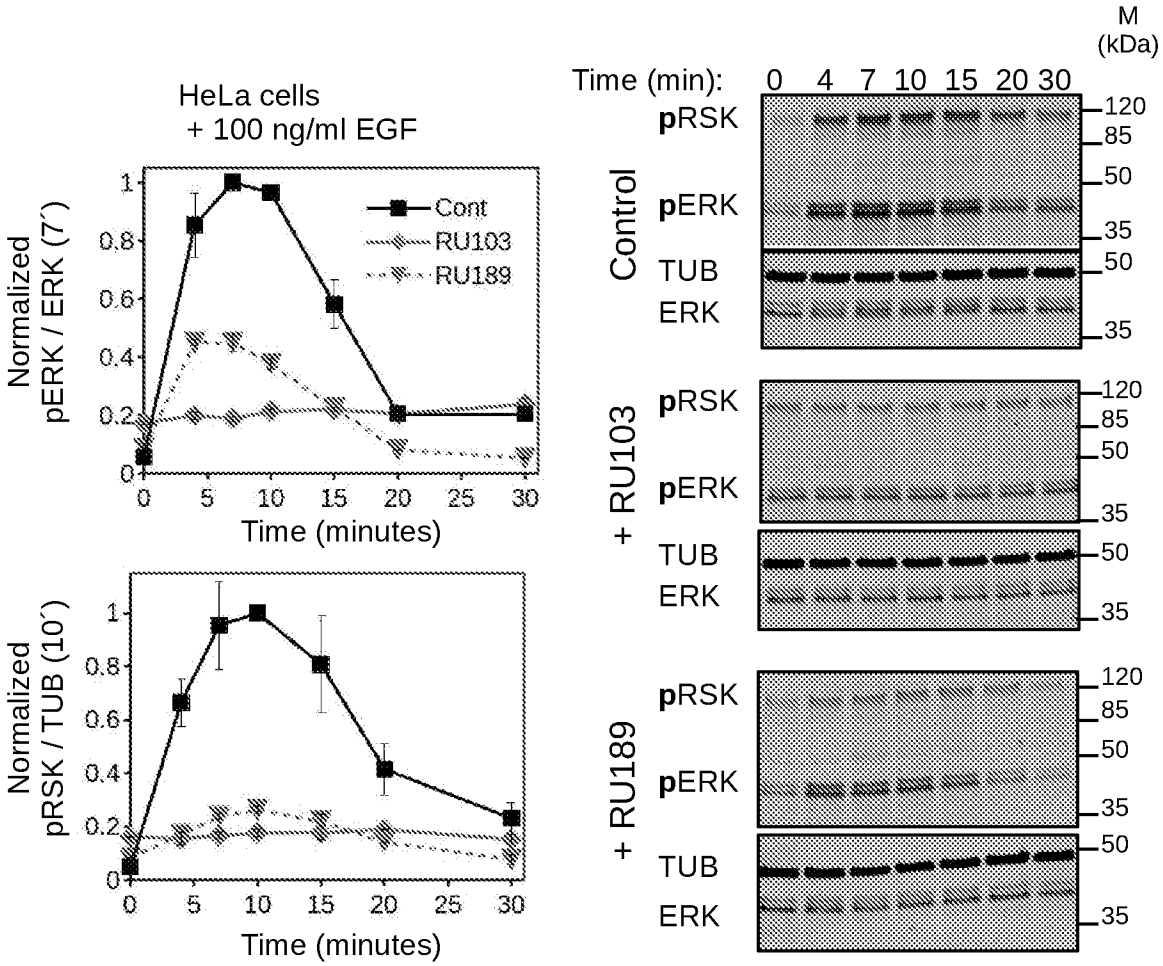

HeLa cells
+ 100 ng/ml EGF
Normalized pERK / ERK (7′)
Cont
RU103
RU189
Time (minutes)
Normalized pRSK / TUB (10′)
Time (minutes)
Time (min): 0 4 7 10 15 20 30
M (kDa)
Control
+ RU103
+ RU189
pRSK
pERK
TUB
ERK
120
85
50
35

FIGURE 9-2

C

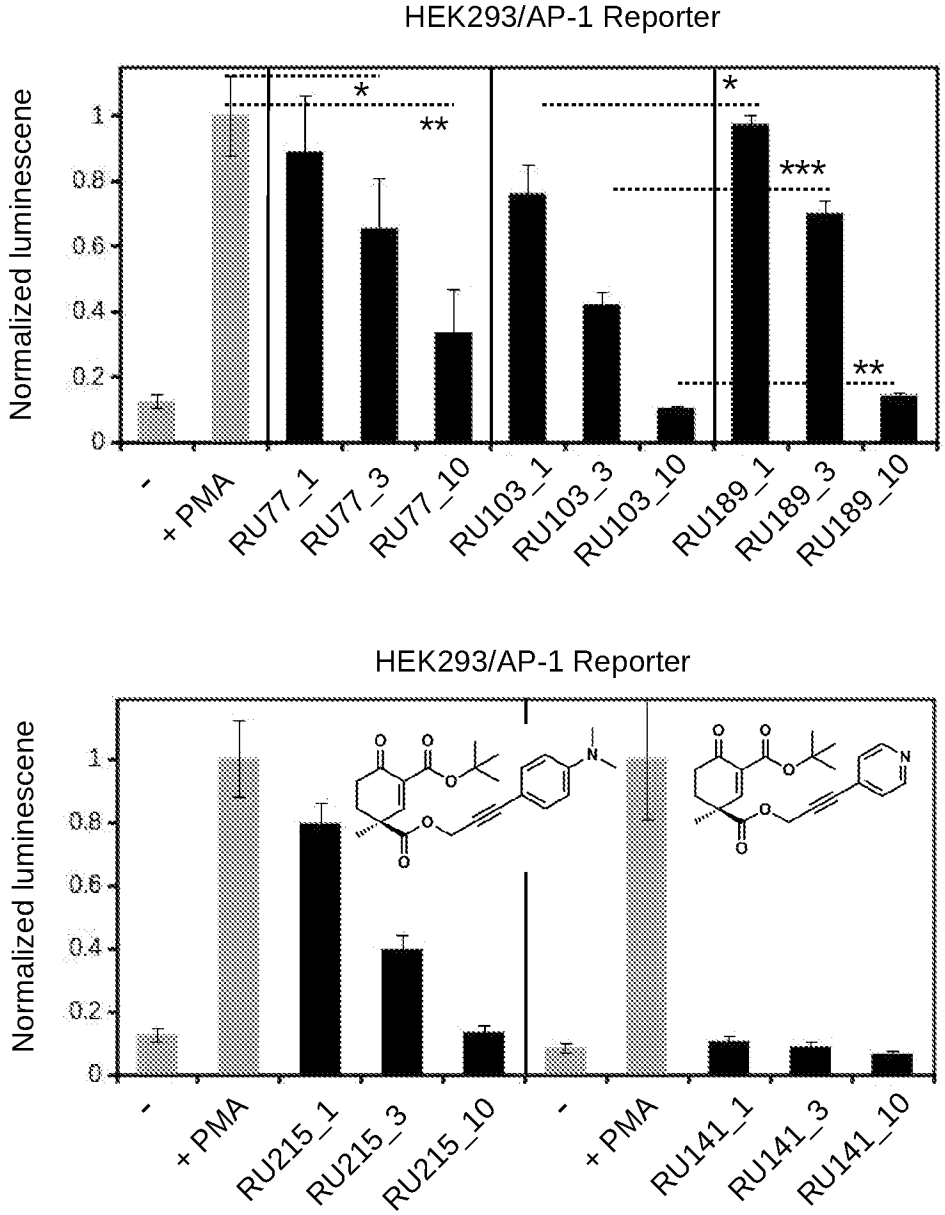

HEK293/AP-1 Reporter
Normalized luminescene
0
0.2
0.4
0.6
0.8
1
*
**
*
***
**
-
+ PMA
RU77_1
RU77_3
RU77_10
RU103_1
RU103_3
RU103_10
RU189_1
RU189_3
RU189_10
HEK293/AP-1 Reporter
Normalized luminescene
0
0.2
0.4
0.6
0.8
1
-
+ PMA
RU215_1
RU215_3
RU215_10
-
+ PMA
RU141_1
RU141_3
RU141_10

FIGURE 9-3

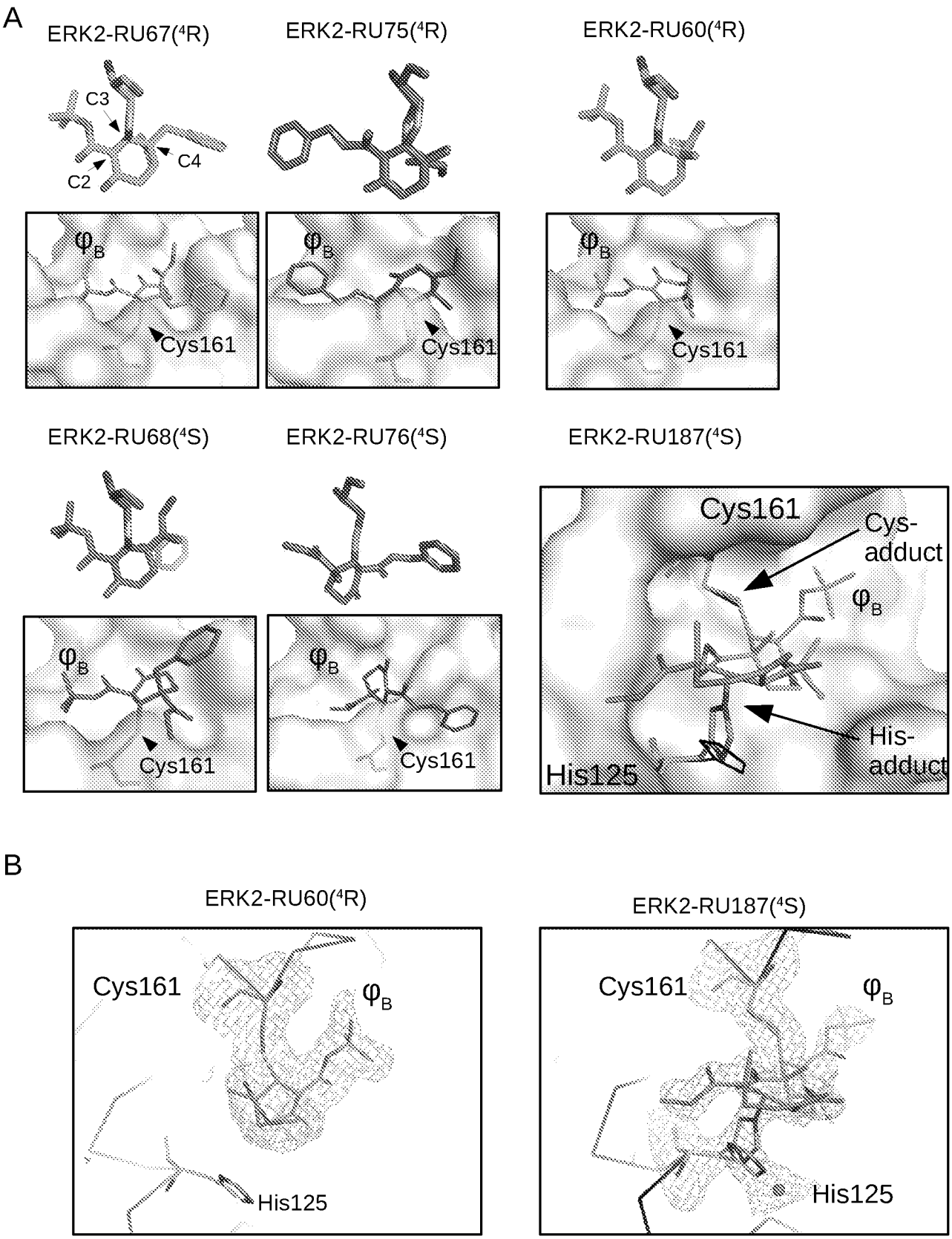
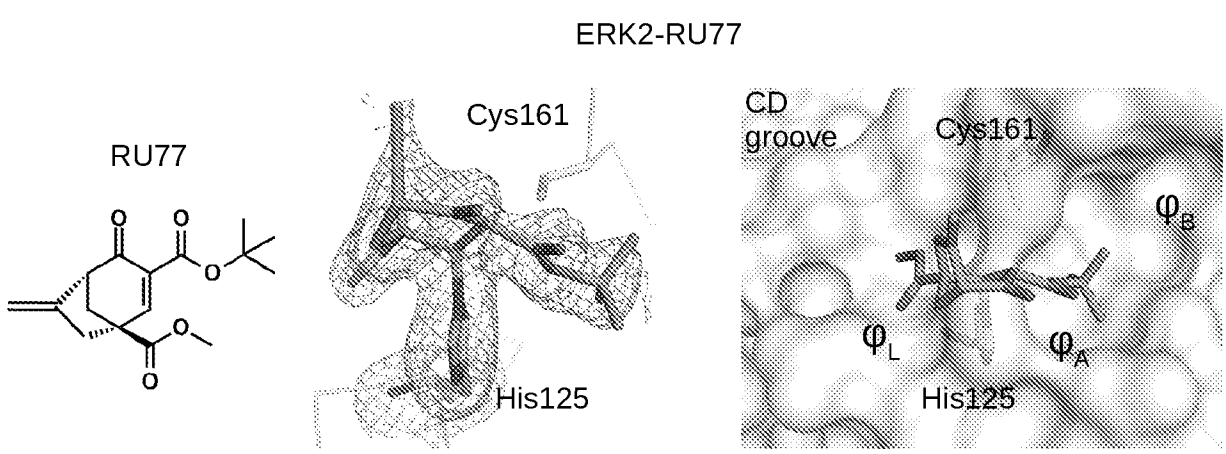

A
ERK2-RU67(4R)
ERK2-RU75(4R)
ERK2-RU60(4R)
C3
C4
C2
φB
Cys161
ERK2-RU68(4S)
ERK2-RU76(4S)
ERK2-RU187(4S)
Cys161
Cys-
adduct
His-
adduct
His125
B
ERK2-RU60(4R)
ERK2-RU187(4S)
Cys161
φB
His125

FIGURE 10

A


ERK2-RU77
RU77
Cys161
His125
CD groove
Cys161
φB
φL
φA
His125

B

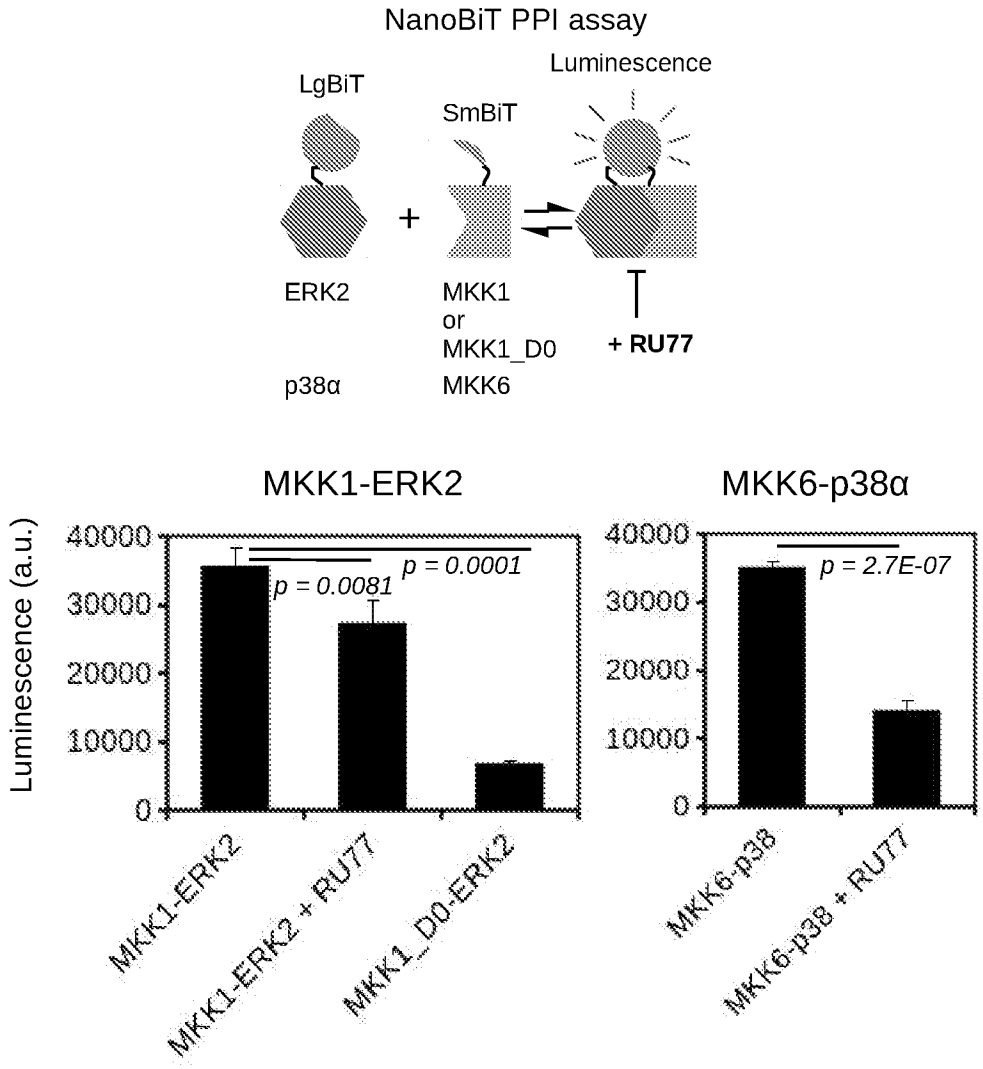
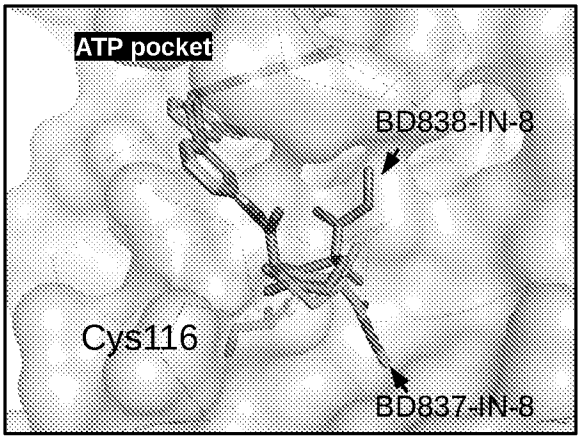
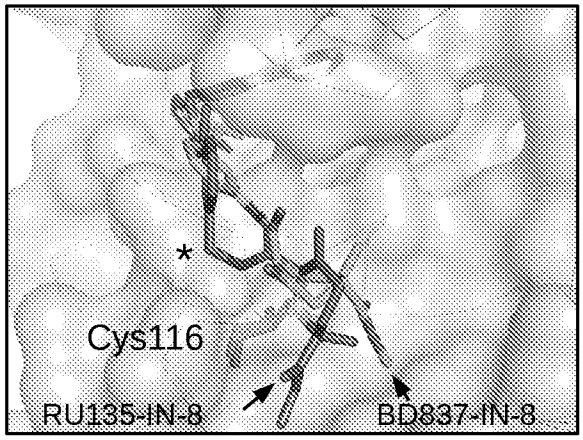

NanoBiT PPI assay
LgBiT
SmBiT
Luminescence
ERK2
MKK1 or MKK1_D0
+ RU77
p38α
MKK6
MKK1-ERK2
MKK6-p38α
Luminescence (a.u.)
40000
30000
20000
10000
0
p = 0.0081
p = 0.0001
p = 2.7E-07
MKK1-ERK2
MKK1-ERK2 + RU77
MKK1_D0-ERK2
MKK6-p38
MKK6-p38 + RU77

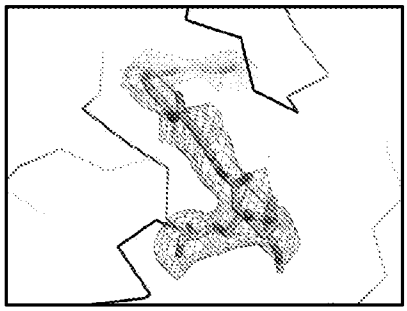
FIGURE 11

JNK1:BD837-IN-8 vs JNK1:BD838-IN-8

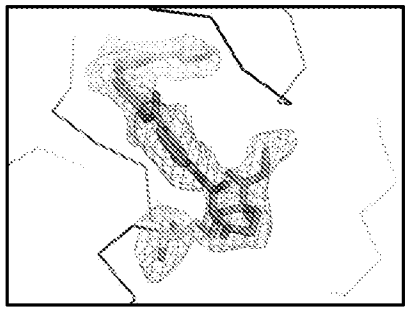


JNK1:BD837-IN-8 vs JNK1:RU135-IN-8

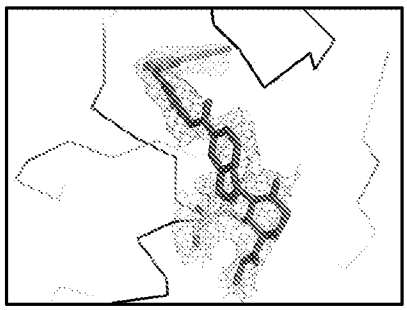




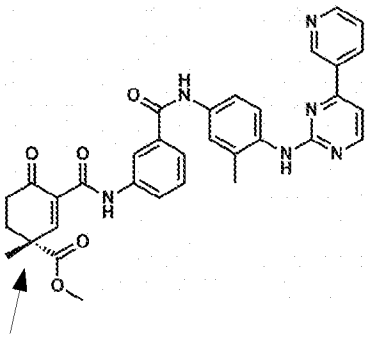
JNK1:BD837-IN-8

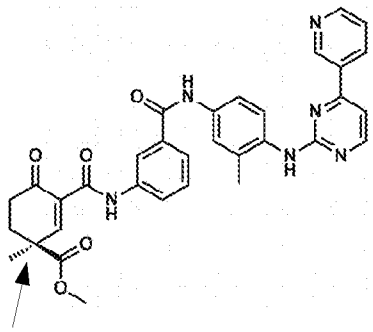


JNK1:BD838-IN-8



JNK1:RU135-IN-8





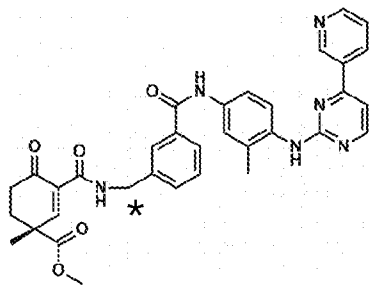


FIGURE 12

A

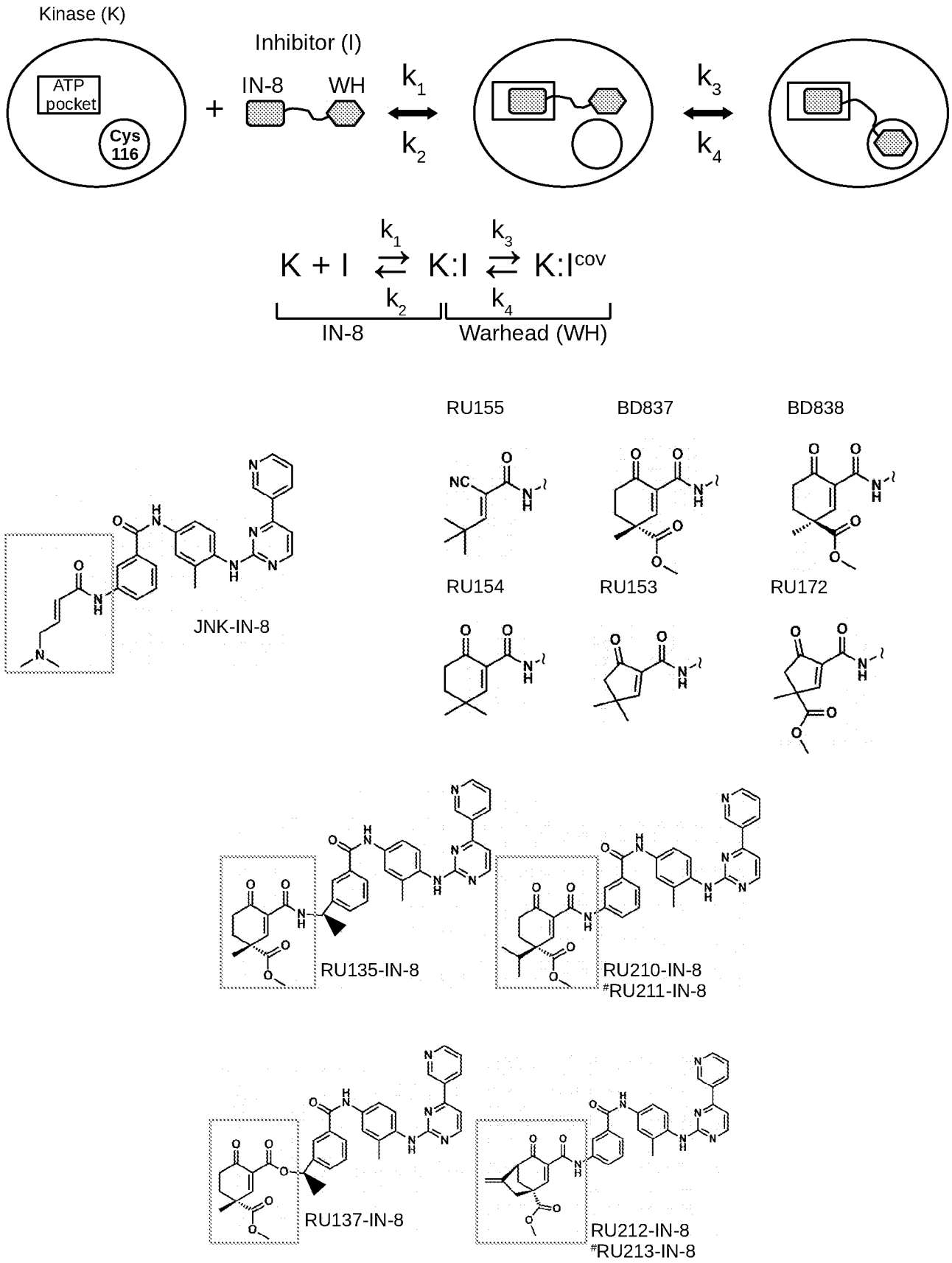

Kinase (K)
Inhibitor (I)
ATP pocket
Cys 116
IN-8
WH
k_1
k_2
k_3
k_4
$K + I \rightleftarrows K:I \rightleftarrows K:I^{cov}$
IN-8
Warhead (WH)
JNK-IN-8
RU155
BD837
BD838
RU154
RU153
RU172
RU135-IN-8
RU210-IN-8
#RU211-IN-8
RU137-IN-8
RU212-IN-8
#RU213-IN-8

FIGURE 13-1

B

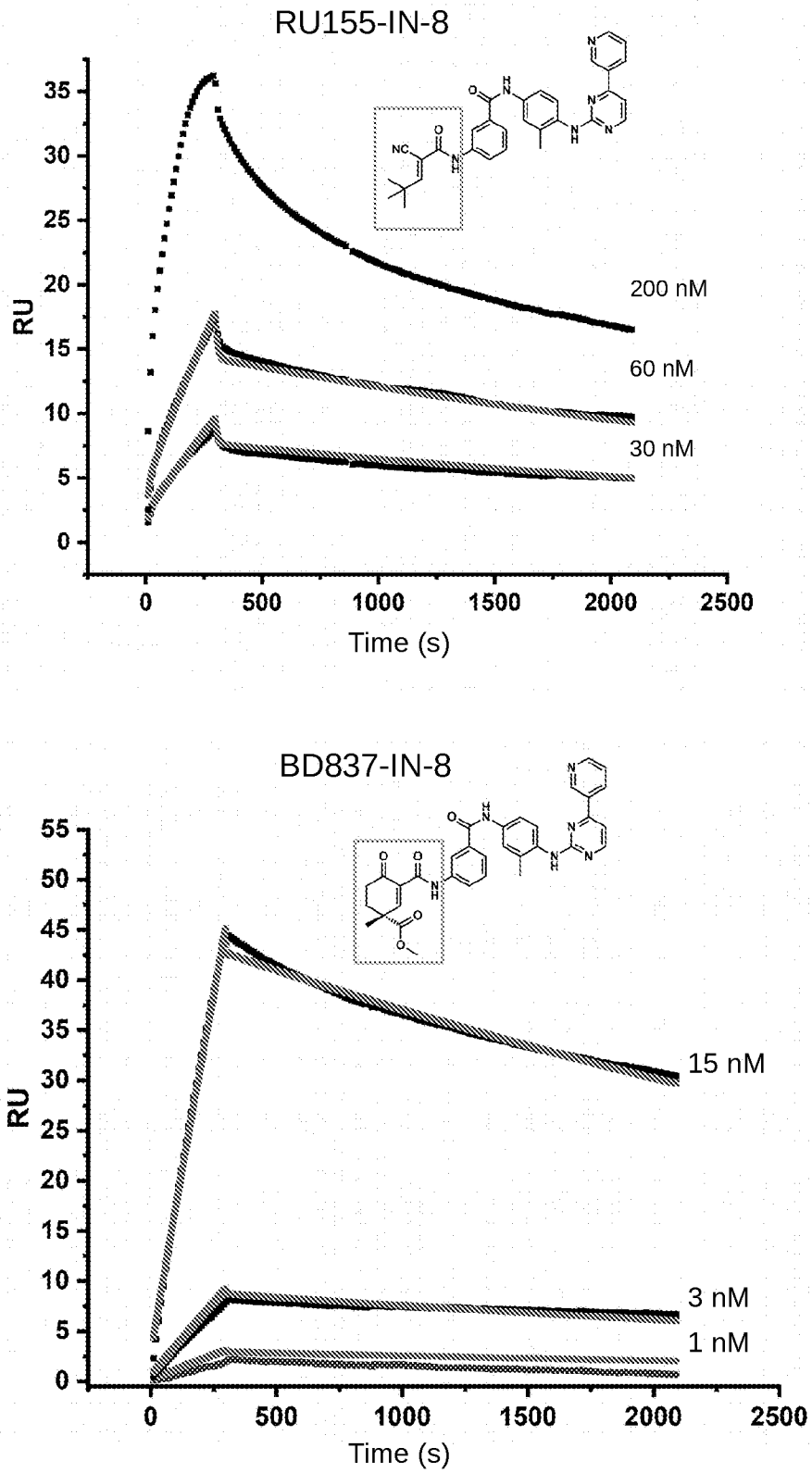

RU155-IN-8
RU
Time (s)
200 nM
60 nM
30 nM
BD837-IN-8
RU
Time (s)
15 nM
3 nM
1 nM

FIGURE 13-2

C

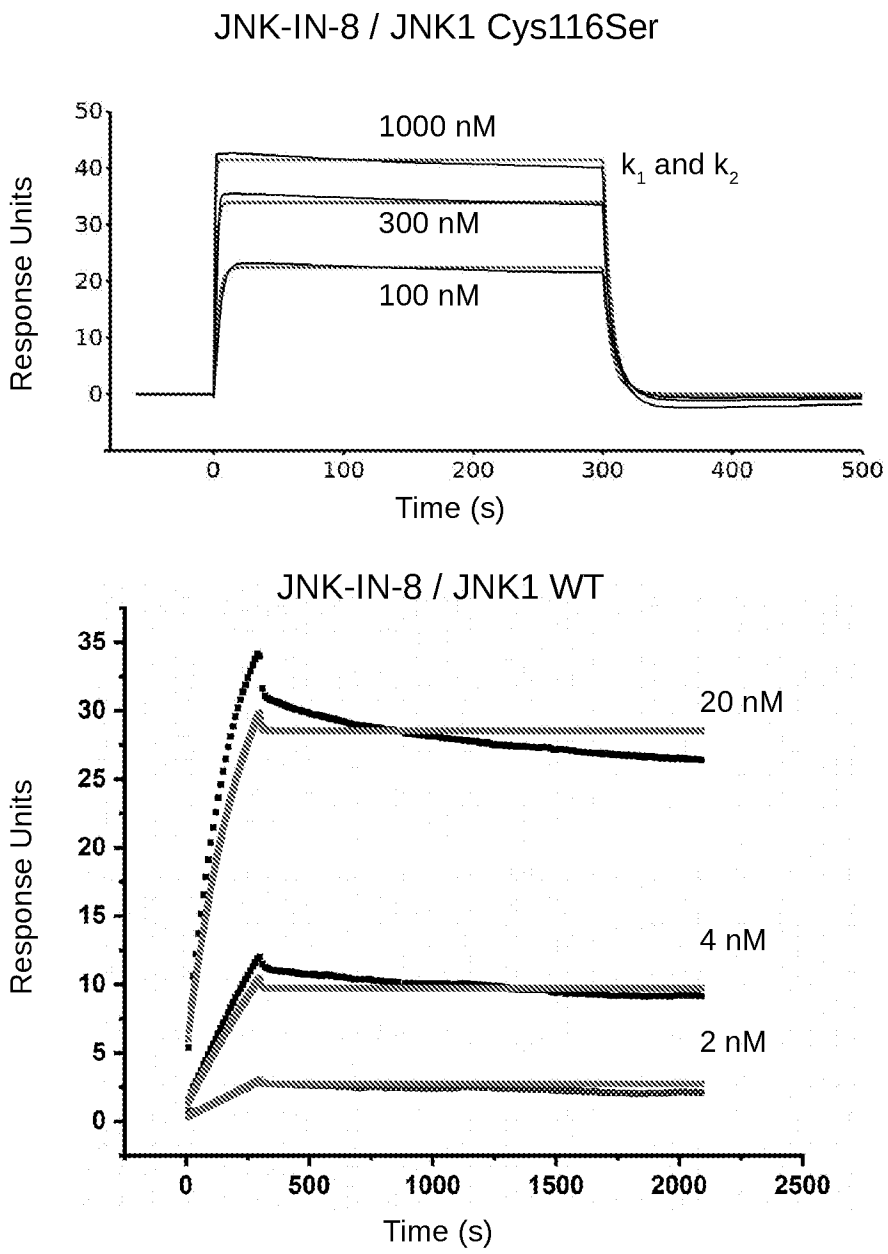

JNK-IN-8 / JNK1 Cys116Ser
1000 nM
k_1 and k_2
300 nM
100 nM
Response Units
Time (s)
JNK-IN-8 / JNK1 WT
20 nM
4 nM
2 nM
Response Units
Time (s)

FIGURE 13-3

D

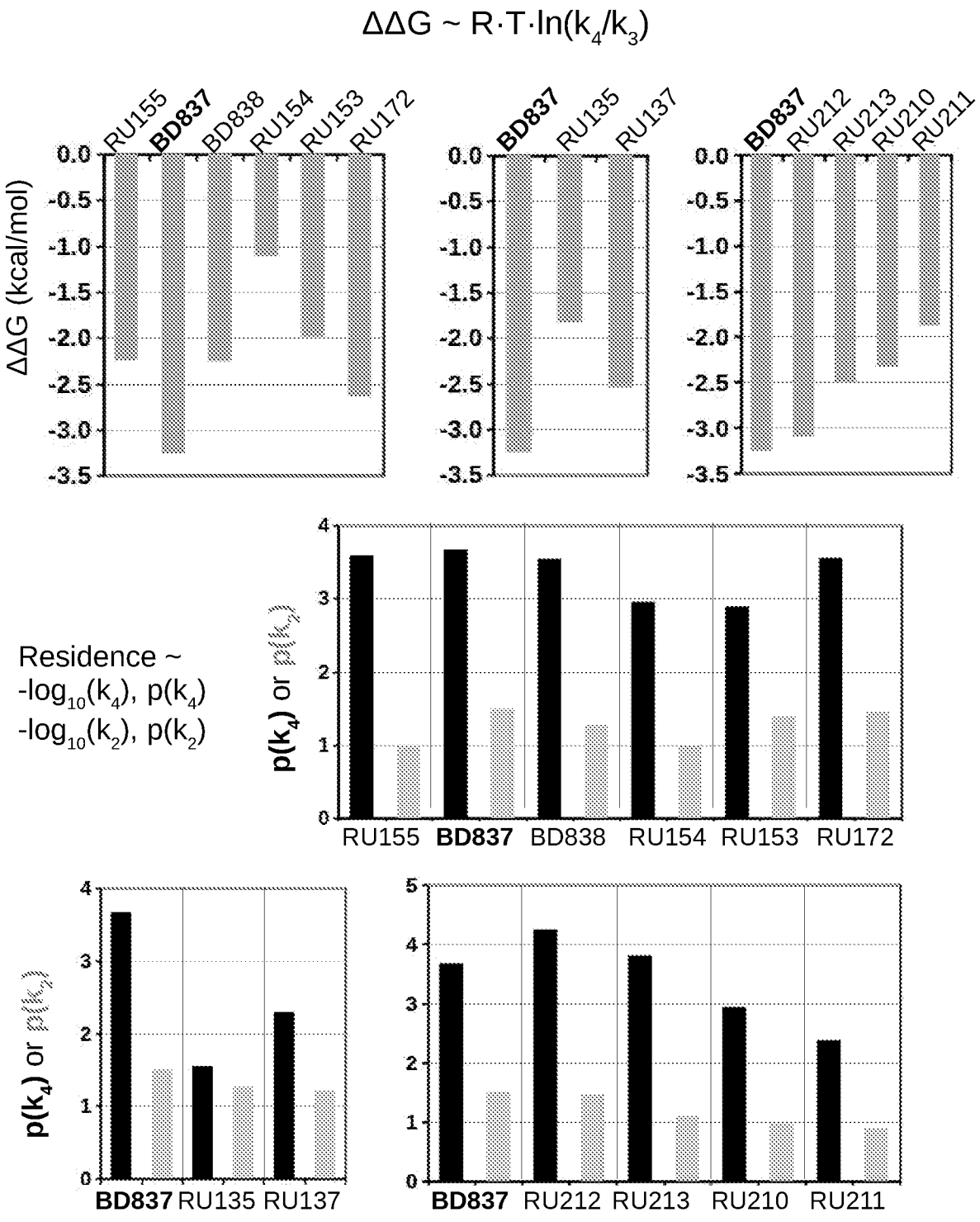

ΔΔG ~ R·T·ln(k4/k3)
RU155
BD837
BD838
RU154
RU153
RU172
BD837
RU135
RU137
BD837
RU212
RU213
RU210
RU211
ΔΔG (kcal/mol)
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
Residence ~
-log10(k4), p(k4)
-log10(k2), p(k2)
p(k4) or p(k2)
RU155 BD837 BD838 RU154 RU153 RU172
BD837 RU135 RU137
BD837 RU212 RU213 RU210 RU211

FIGURE 13-4

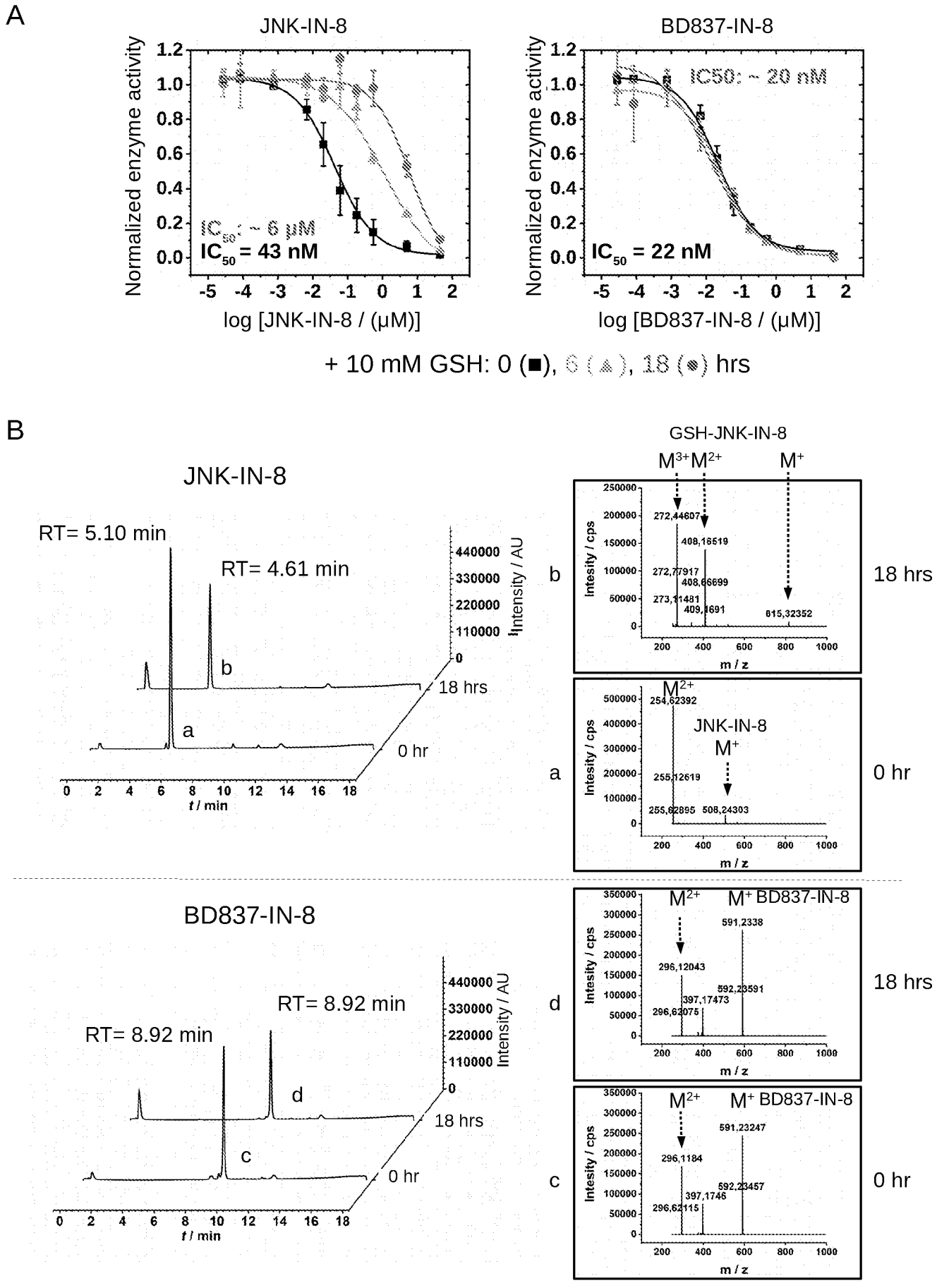
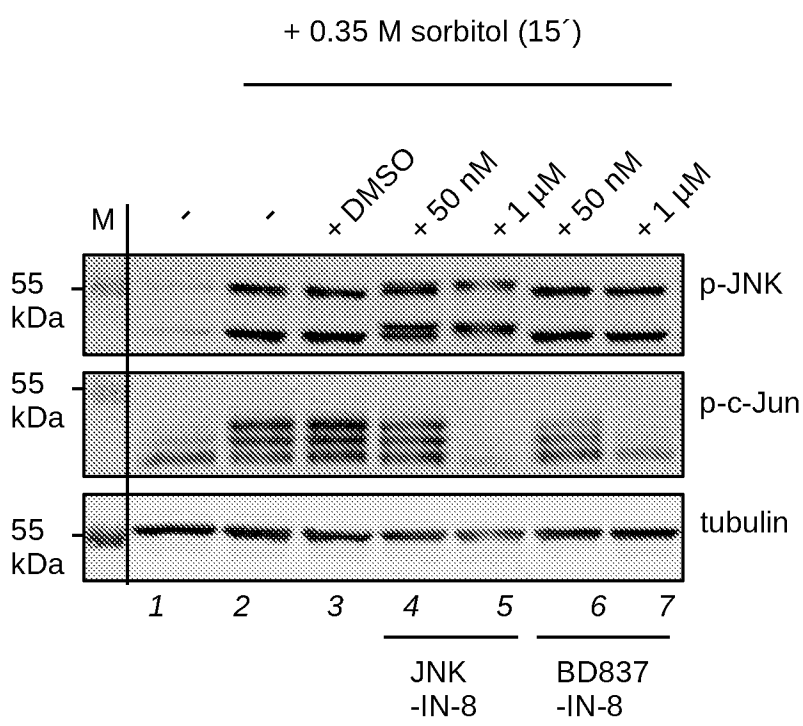

A
JNK-IN-8
BD837-IN-8
Normalized enzyme activity
IC50: ~ 6 µM
IC50 = 43 nM
IC50: ~ 20 nM
IC50 = 22 nM
log [JNK-IN-8 / (µM)]
log [BD837-IN-8 / (µM)]
+ 10 mM GSH: 0 (■), 6 (▲), 18 (●) hrs
B
JNK-IN-8
RT= 5.10 min
RT= 4.61 min
Intensity / AU
t / min
18 hrs
0 hr
GSH-JNK-IN-8
M3+ M2+
M+
Intesity / cps
m / z
272,44607
408,16519
272,77917
408,66699
273,11481
409,1691
615,32352
254,62392
255,12619
255,62895
508,24303
BD837-IN-8
RT= 8.92 min
RT= 8.92 min
M2+
M+ BD837-IN-8
591,2338
296,12043
592,23591
397,17473
296,62075
591,23247
296,1184
592,23457
397,1746
296,62115

FIGURE 14

A



B

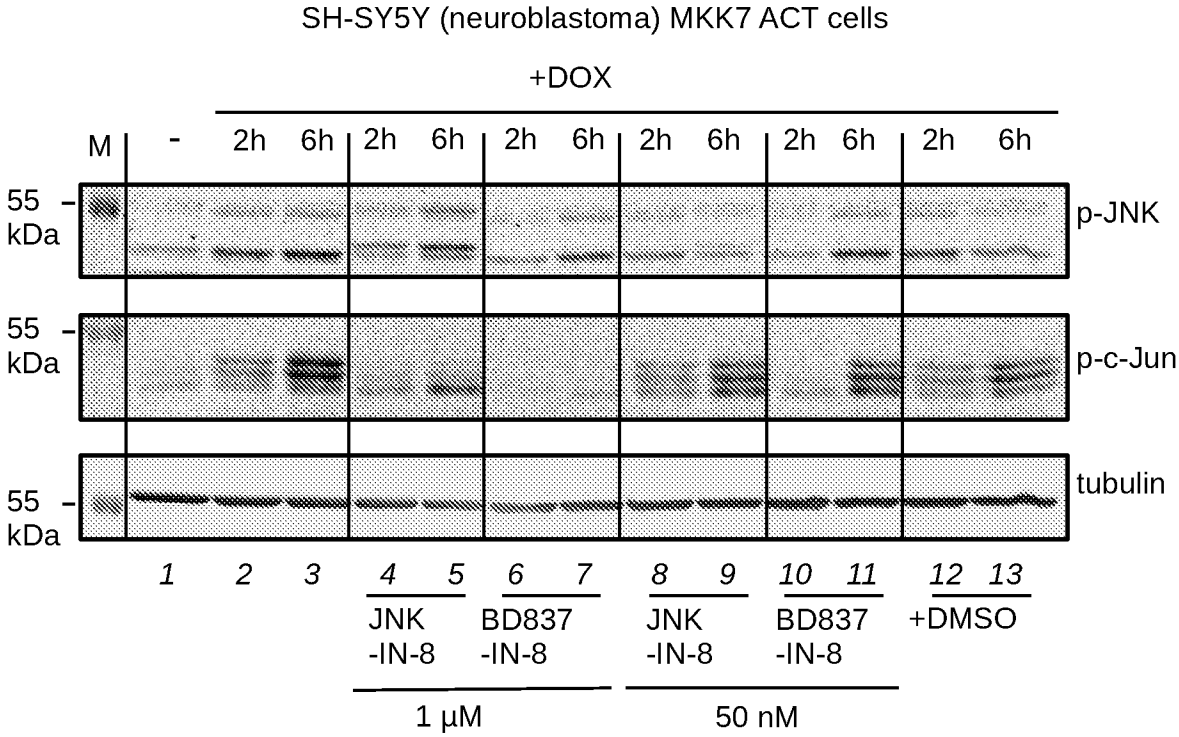
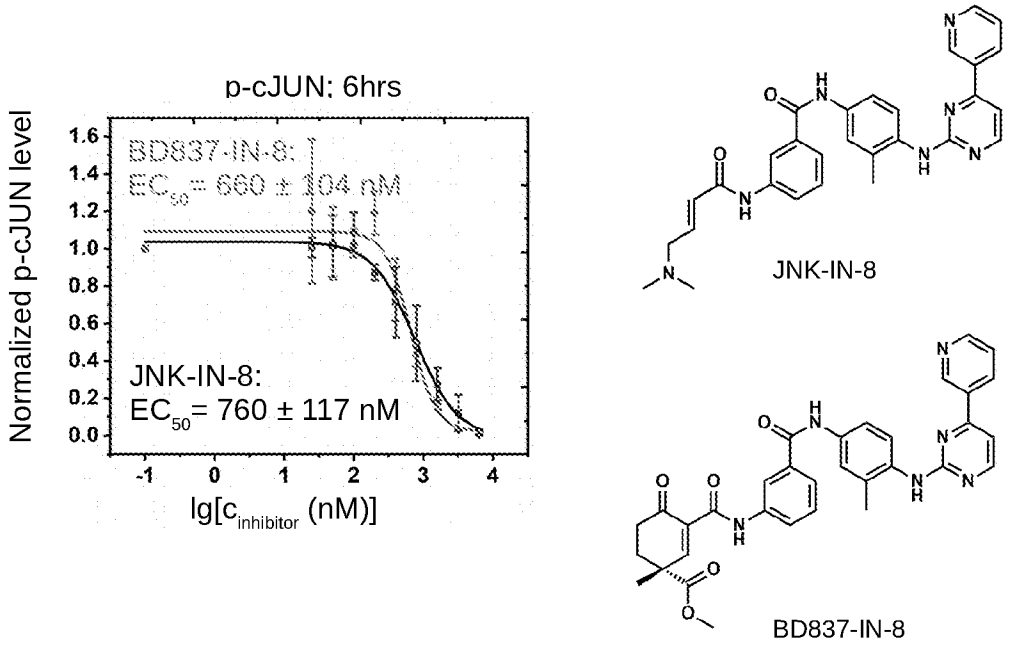
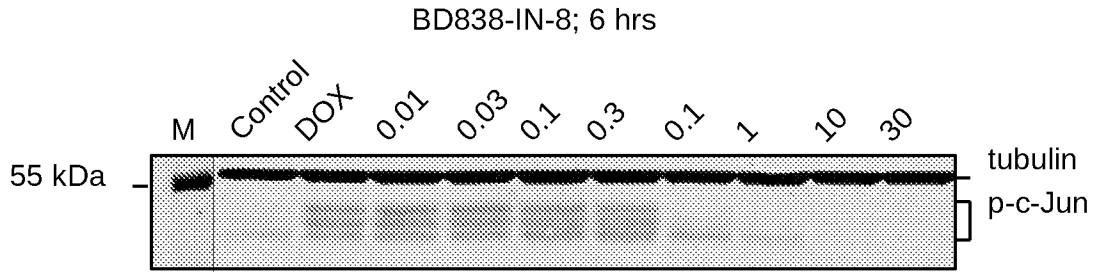


FIGURE 15-1

C


p-cJUN: 6hrs
Normalized p-cJUN level
BD837-IN-8:
EC50= 660 ± 104 nM
JNK-IN-8:
EC50= 760 ± 117 nM
lg[cinhibitor (nM)]
JNK-IN-8
BD837-IN-8


BD838-IN-8; 6 hrs
M
Control
DOX
0.01
0.03
0.1
0.3
0.1
1
10
30
55 kDa
tubulin
p-c-Jun

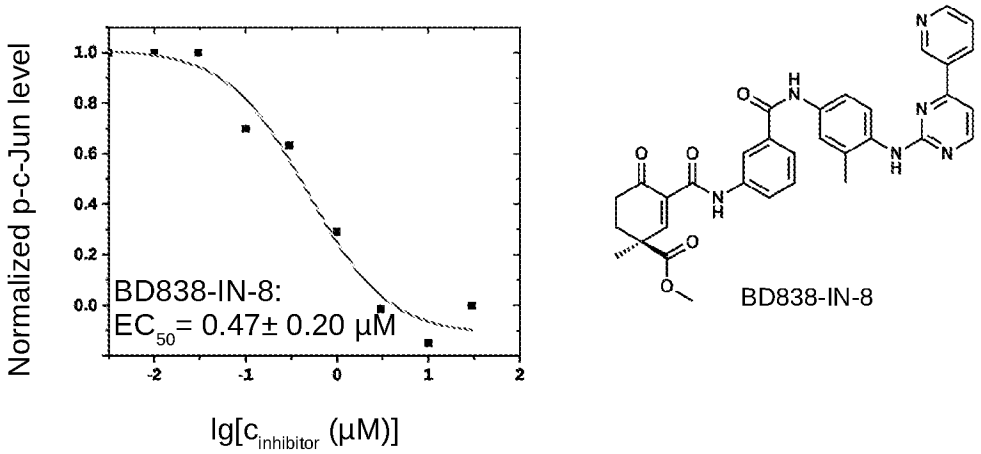

Normalized p-c-Jun level
BD838-IN-8:
EC50= 0.47± 0.20 µM
lg[cinhibitor (µM)]
BD838-IN-8

FIGURE 15-2

D

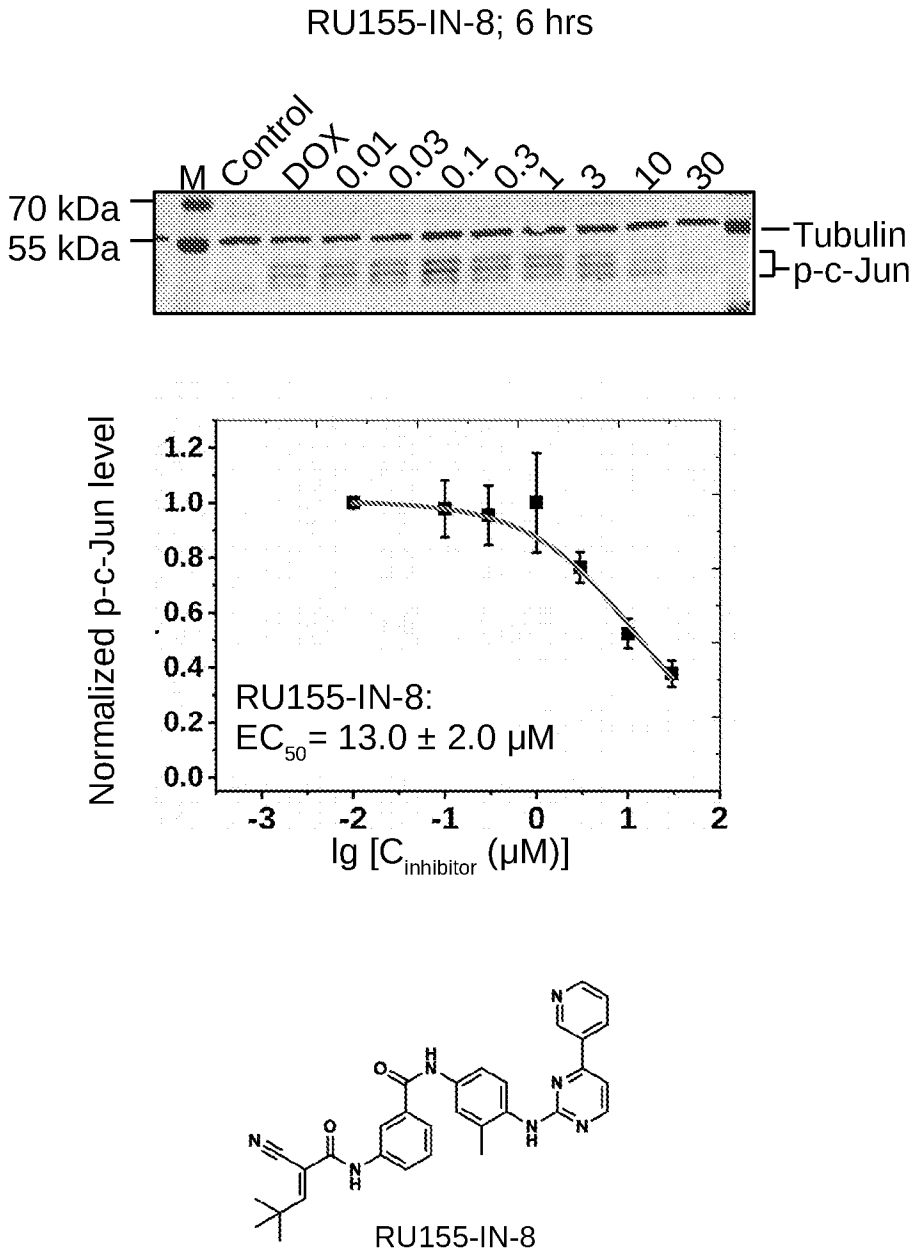

RU155-IN-8; 6 hrs
M
Control
DOX
0.01
0.03
0.1
0.3
1
3
10
30
70 kDa
55 kDa
Tubulin
p-c-Jun
Normalized p-c-Jun level
RU155-IN-8:
EC50= 13.0 ± 2.0 µM
lg [Cinhibitor (µM)]
RU155-IN-8

FIGURE 15-3

E

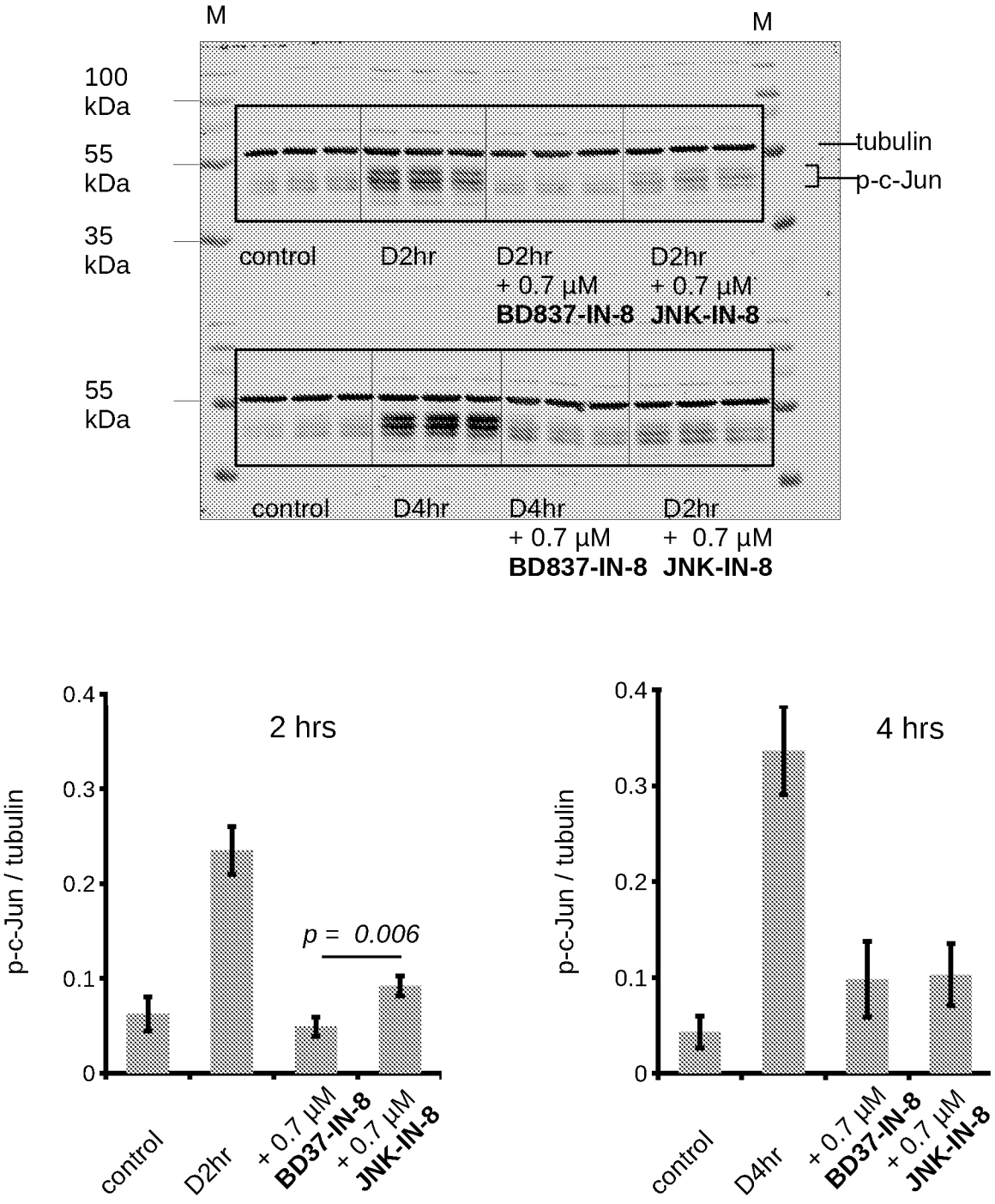

M
M
100 kDa
55 kDa
35 kDa
tubulin
p-c-Jun
control
D2hr
D2hr + 0.7 µM BD837-IN-8
D2hr + 0.7 µM JNK-IN-8
55 kDa
control
D4hr
D4hr + 0.7 µM BD837-IN-8
D2hr + 0.7 µM JNK-IN-8
2 hrs
p-c-Jun / tubulin
0.4
0.3
0.2
0.1
0
p = 0.006
control
D2hr
+ 0.7 µM BD37-IN-8
+ 0.7 µM JNK-IN-8
4 hrs
p-c-Jun / tubulin
0.4
0.3
0.2
0.1
0
control
D4hr
+ 0.7 µM BD37-IN-8
+ 0.7 µM JNK-IN-8

FIGURE 15-4

A

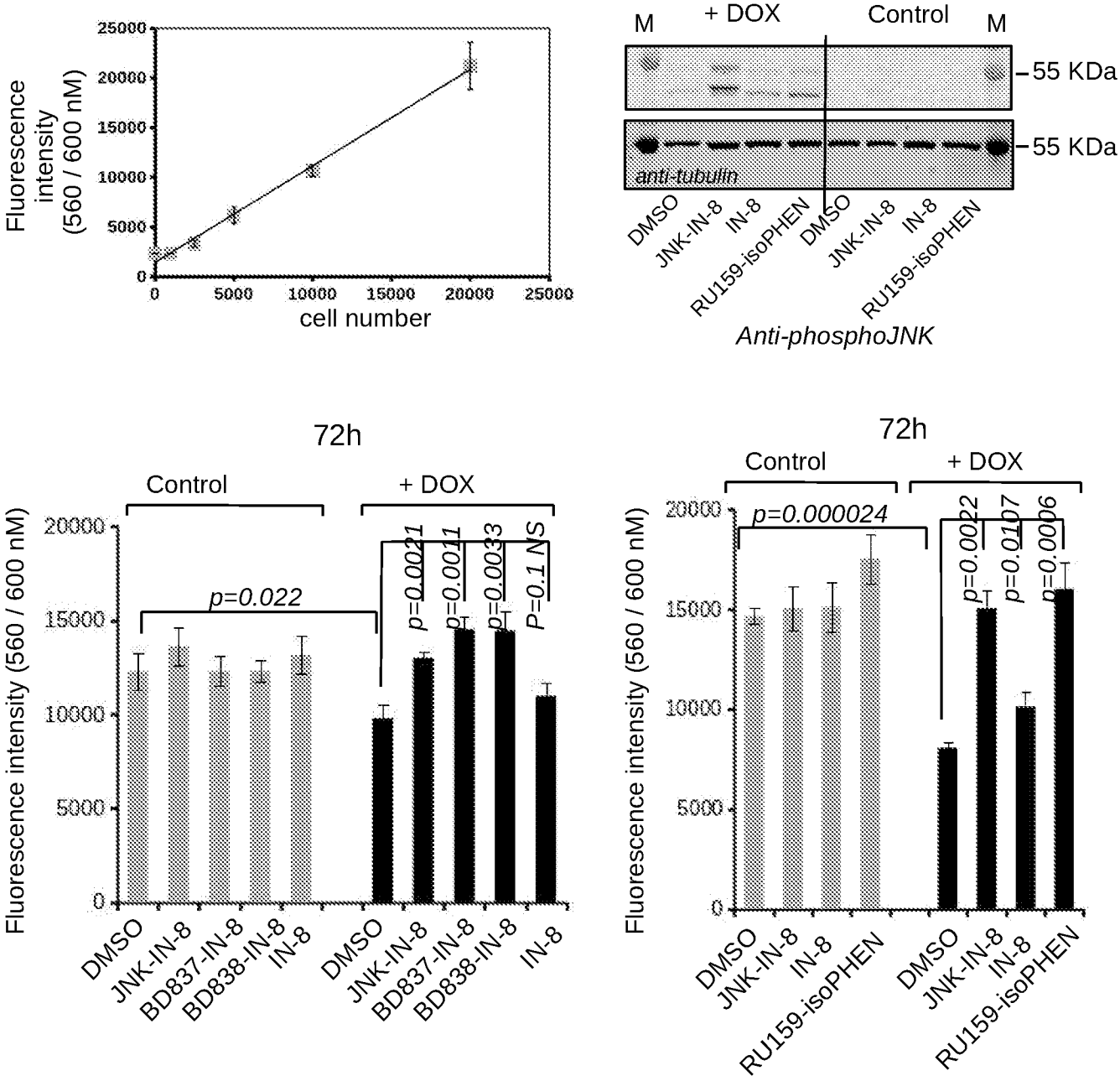

Fluorescence intensity (560 / 600 nM)
cell number
M
+ DOX
Control
M
55 KDa
55 KDa
anti-tubulin
DMSO
JNK-IN-8
IN-8
RU159-isoPHEN
Anti-phosphoJNK
72h
Control
+ DOX
p=0.022
p=0.0021
p=0.0011
p=0.0033
P=0.1 NS
DMSO
JNK-IN-8
BD837-IN-8
BD838-IN-8
IN-8
72h
p=0.000024
p=0.0022
p=0.0107
p=0.0006
RU159-isoPHEN

FIGURE 16-1

B

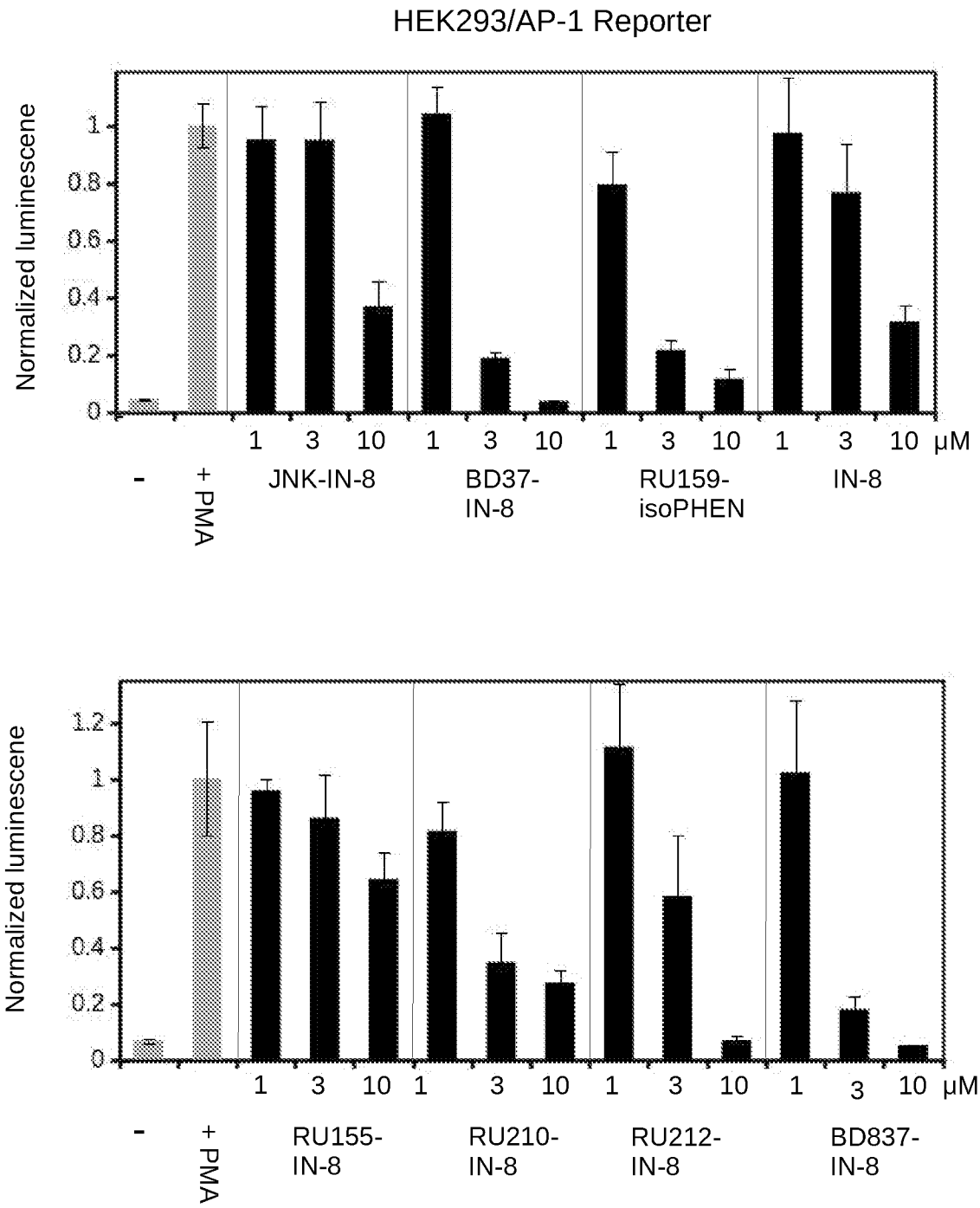

HEK293/AP-1 Reporter
Normalized luminescene
0
0.2
0.4
0.6
0.8
1
1.2
1 3 10 1 3 10 1 3 10 1 3 10 µM
- + PMA JNK-IN-8 BD37-IN-8 RU159-isoPHEN IN-8
1 3 10 1 3 10 1 3 10 1 3 10 µM
- + PMA RU155-IN-8 RU210-IN-8 RU212-IN-8 BD837-IN-8

FIGURE 16-2

A

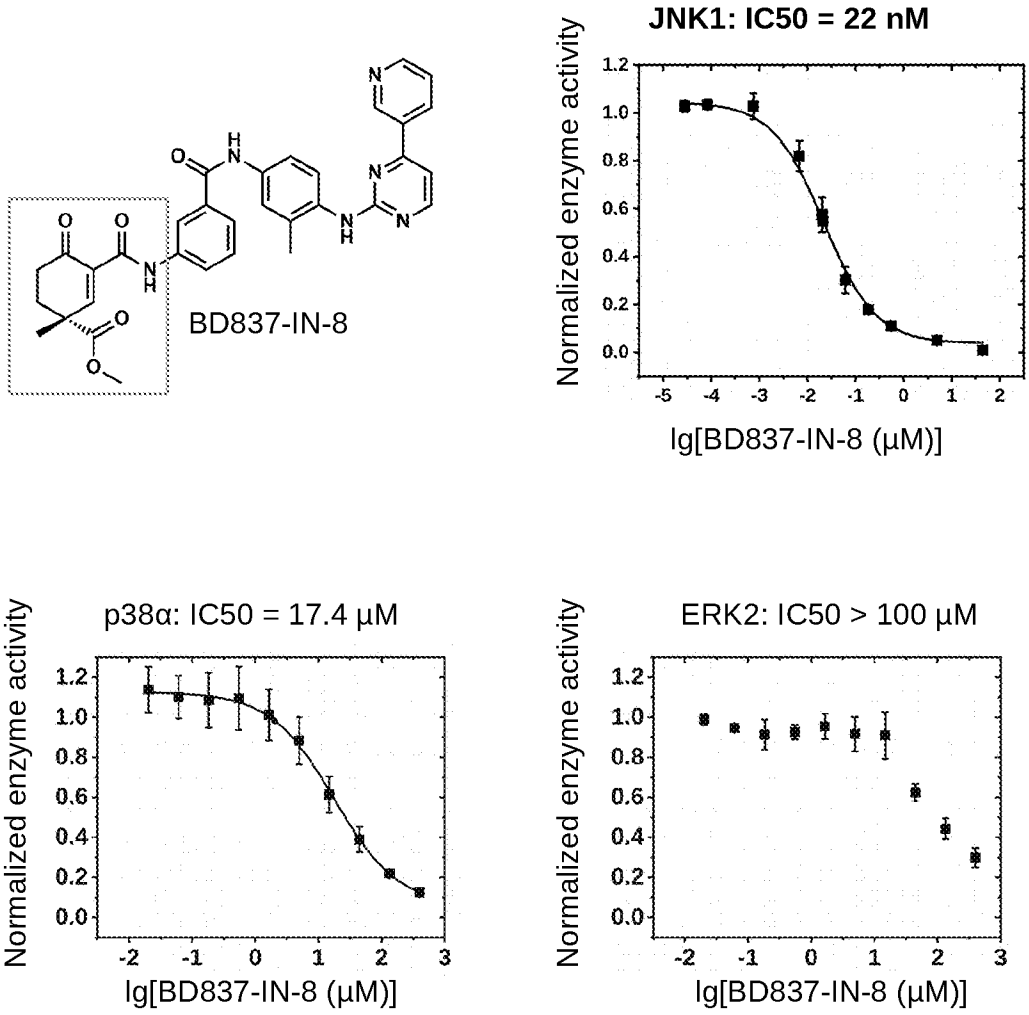
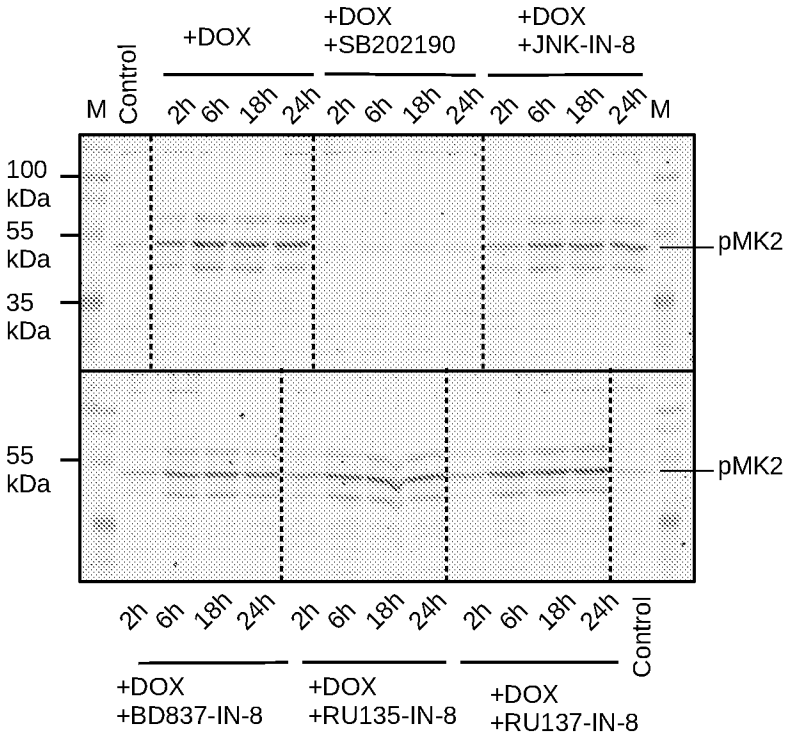
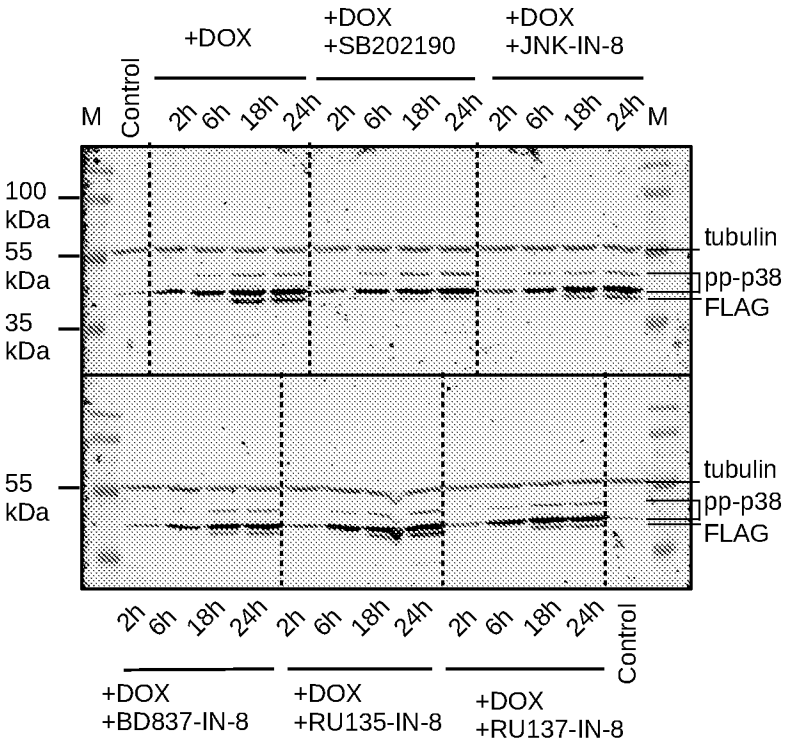

BD837-IN-8
JNK1: IC50 = 22 nM
Normalized enzyme activity
lg[BD837-IN-8 (μM)]
p38α: IC50 = 17.4 μM
Normalized enzyme activity
lg[BD837-IN-8 (μM)]
ERK2: IC50 > 100 μM
Normalized enzyme activity
lg[BD837-IN-8 (μM)]

FIGURE 17-1

B



anti-pMK2



anti-tubilin, anti-pp-p38, anti-FLAG

FIGURE 17-2

C

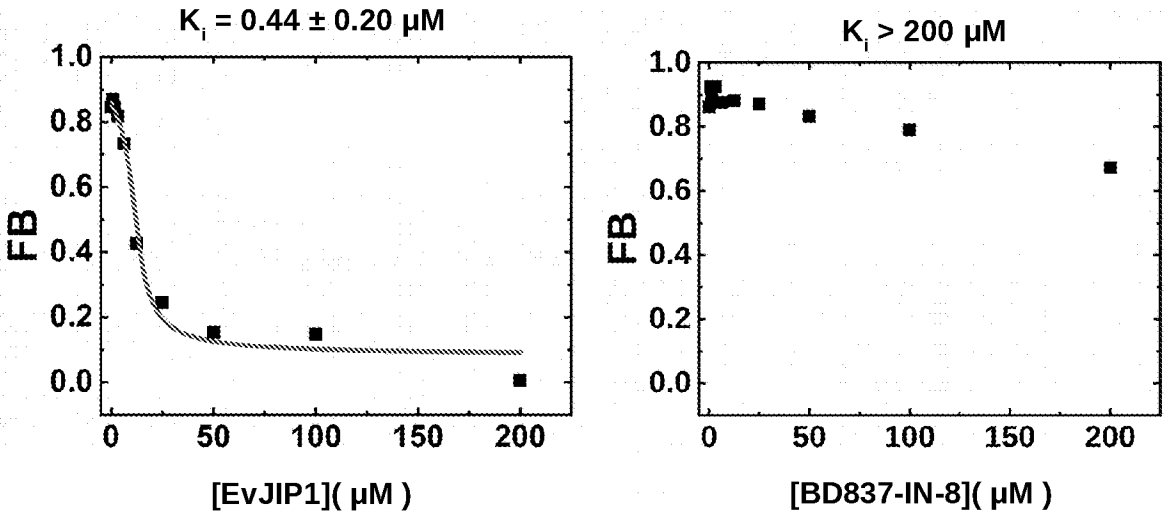
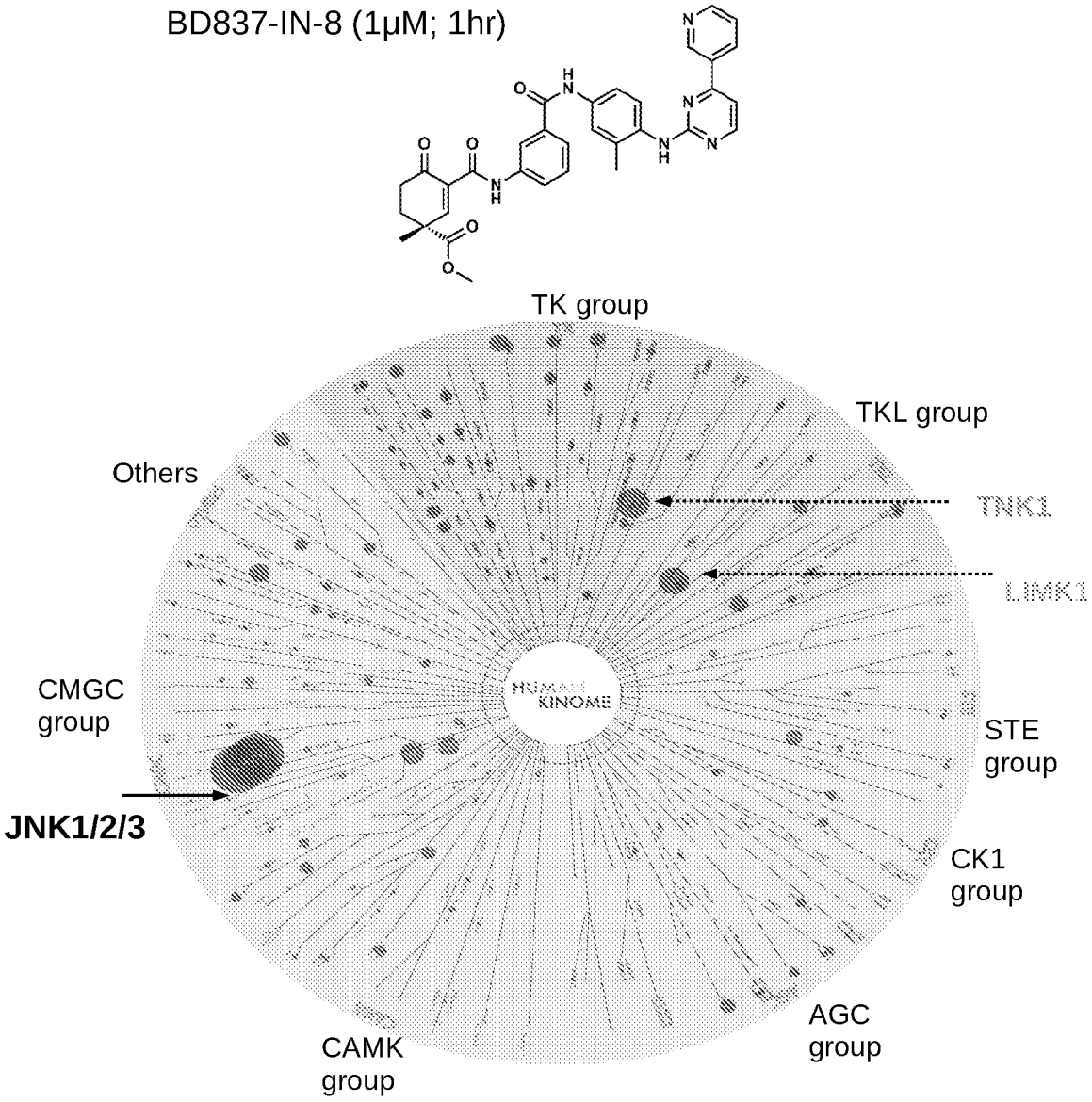

K$_i$ = 0.44 ± 0.20 µM
FB
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
[EvJIP1](µM)
K$_i$ > 200 µM
FB
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
[BD837-IN-8](µM)

FIGURE 17-3


BD837-IN-8 (1μM; 1hr)
TK group
TKL group
Others
TNK1
LIMK1
CMGC group
JNK1/2/3
STE group
CK1 group
AGC group
CAMK group

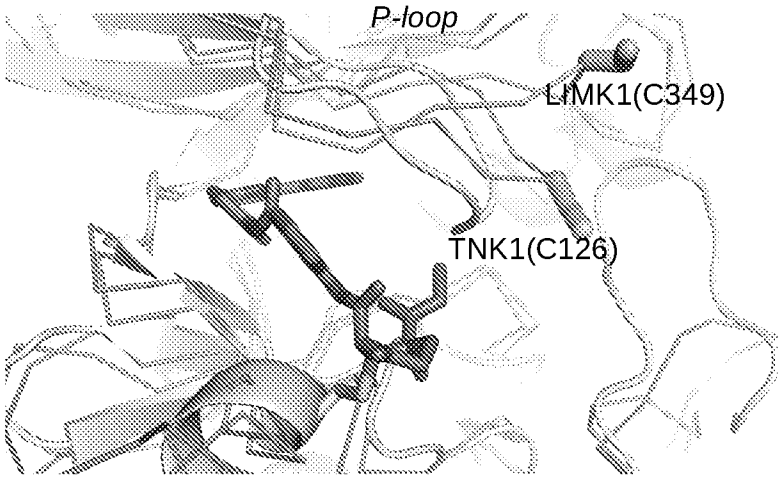
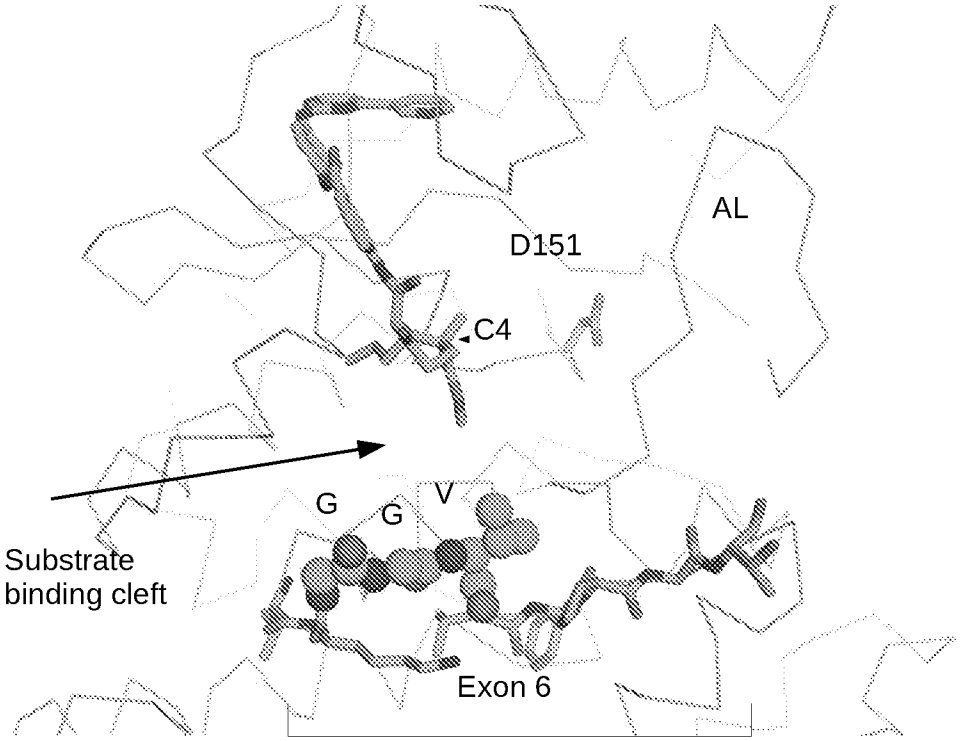

P-loop
LIMK1(C349)
TNK1(C126)

FIGURE 18

A

JNK1−BD837-IN-8



JNK isoforms

```
JNK2a2   203-KENVDIWSVGCIMGELVKGCVIFQGTDHIDQWNKVIEQLGTP-243
JNK1b1   203-KENVDIWSVGCIMGEMIKGGVLFPGTDHIDQWNKVIEQLGTP-243
JNK3a1   241-KENVDIWSVGCIMGEMVRHKILFPGRDYIDQWNKVIEQLGTP-281
         ***************:::  ::* * *:***************
```

B

RU144-IN-8

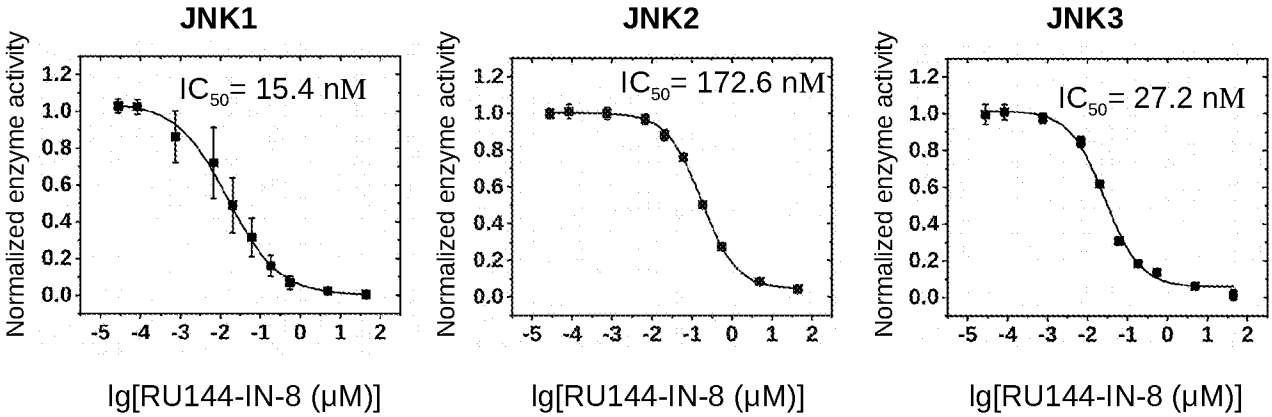


FIGURE 19

A

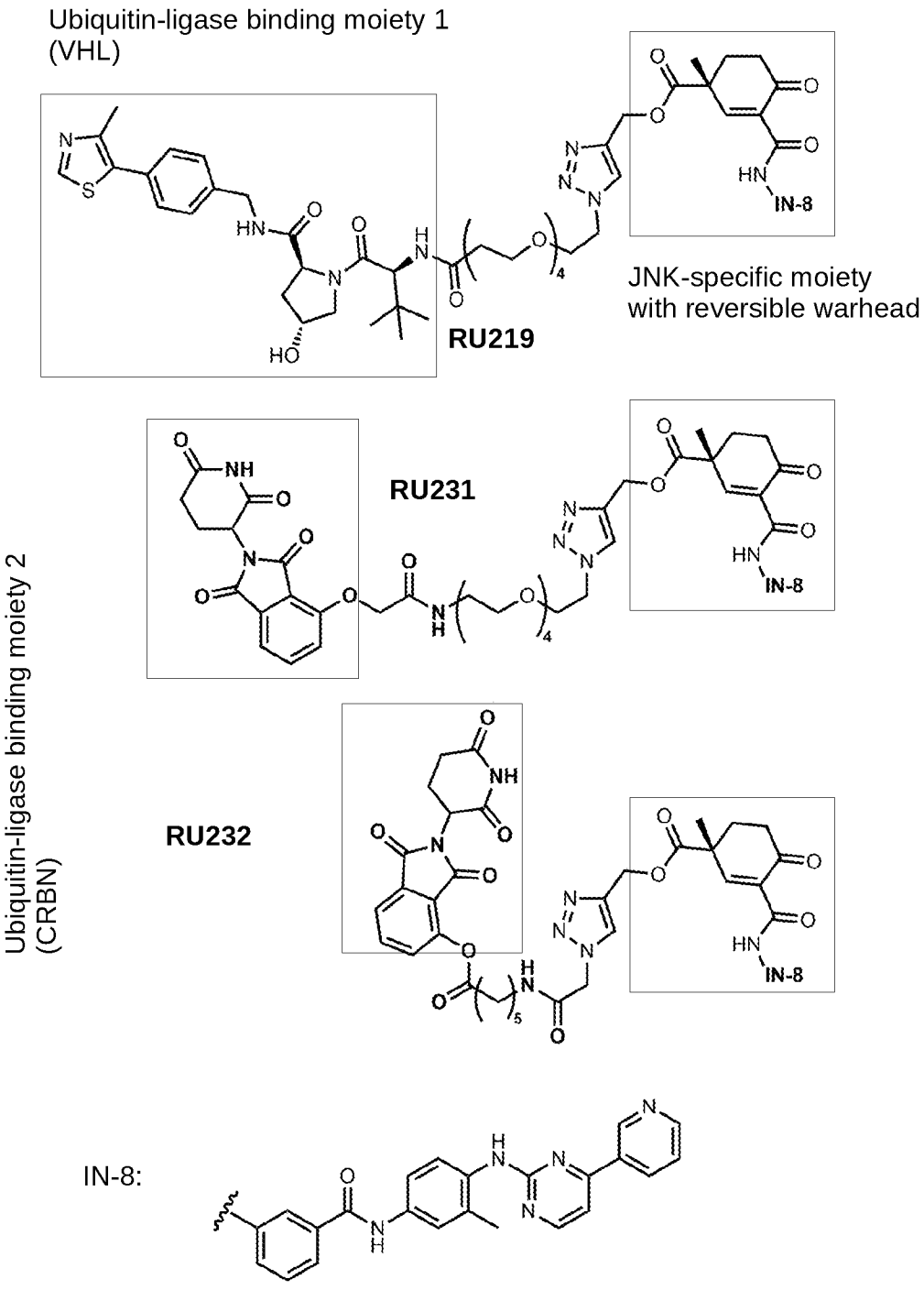

Ubiquitin-ligase binding moiety 1
(VHL)
JNK-specific moiety
with reversible warhead
RU219
RU231
RU232
Ubiquitin-ligase binding moiety 2
(CRBN)
IN-8
IN-8:

FIGURE 20-1

B

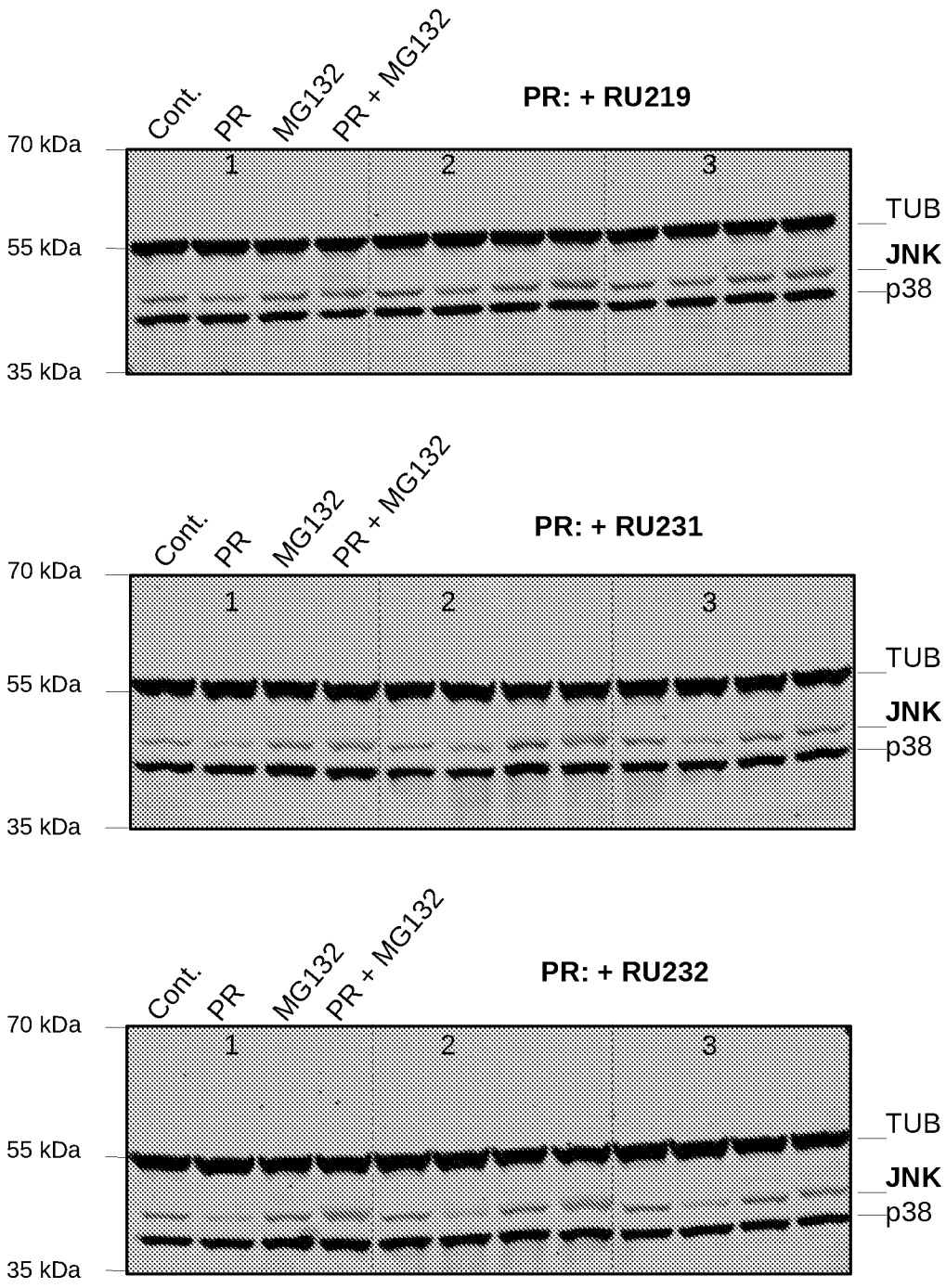

Cont.
PR
MG132
PR + MG132
PR: + RU219
70 kDa
55 kDa
35 kDa
1
2
3
TUB
JNK
p38
Cont.
PR
MG132
PR + MG132
PR: + RU231
70 kDa
55 kDa
35 kDa
1
2
3
TUB
JNK
p38
Cont.
PR
MG132
PR + MG132
PR: + RU232
70 kDa
55 kDa
35 kDa
1
2
3
TUB
JNK
p38

FIGURE 20-2

C

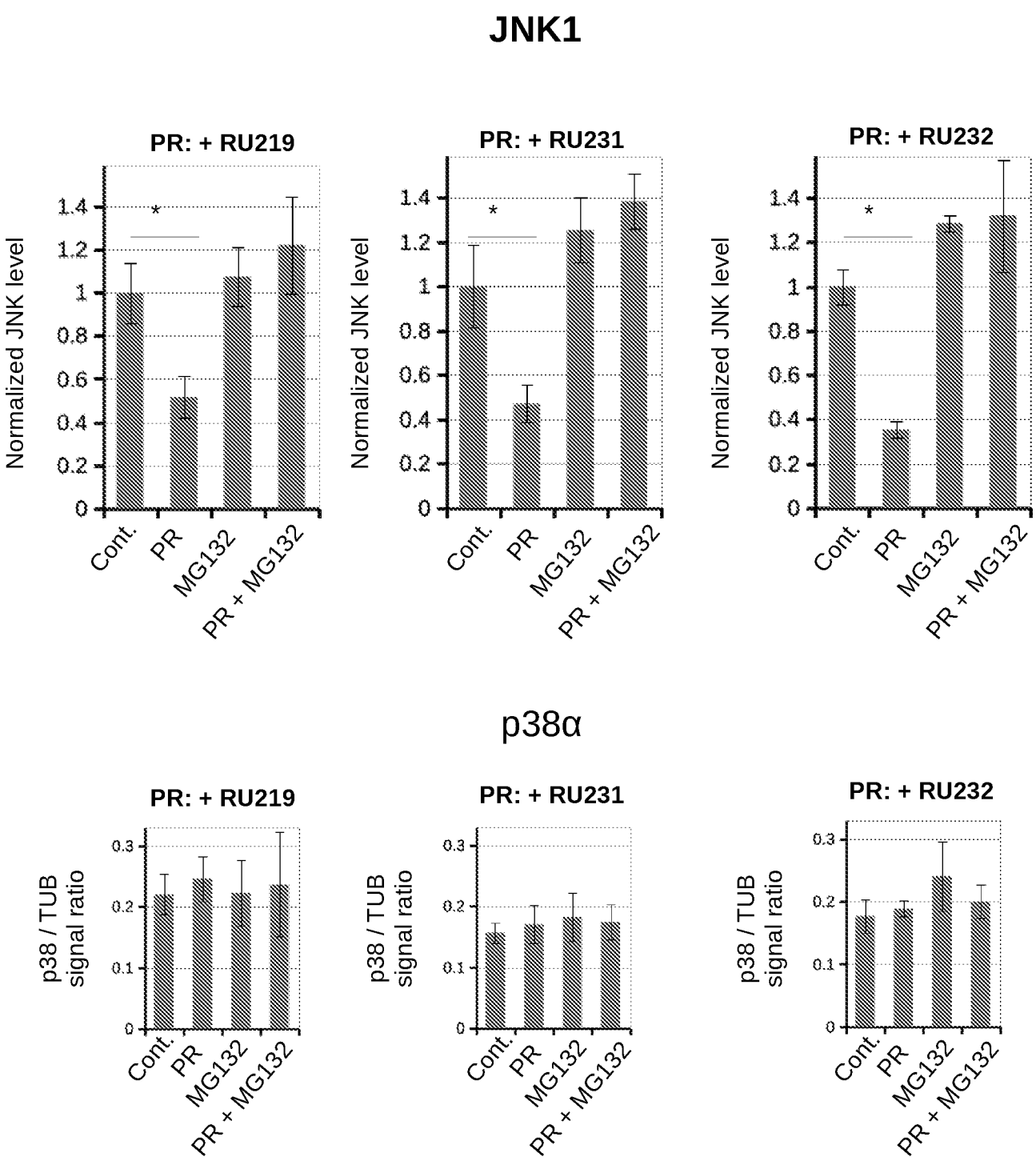
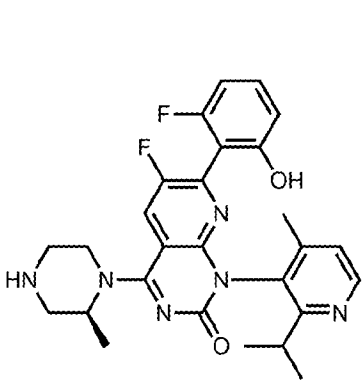
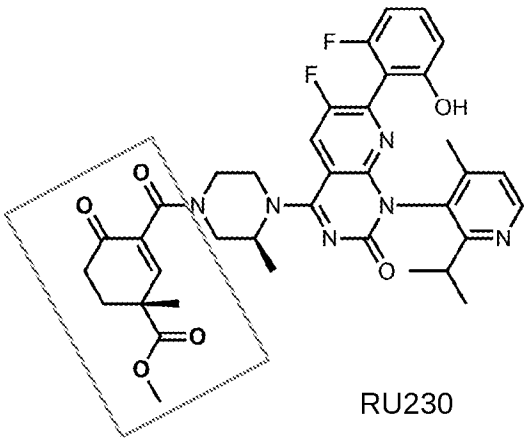

JNK1
PR: + RU219
PR: + RU231
PR: + RU232
Normalized JNK level
0
0.2
0.4
0.6
0.8
1
1.2
1.4
*
Cont.
PR
MG132
PR + MG132
p38α
p38 / TUB signal ratio
0
0.1
0.2
0.3

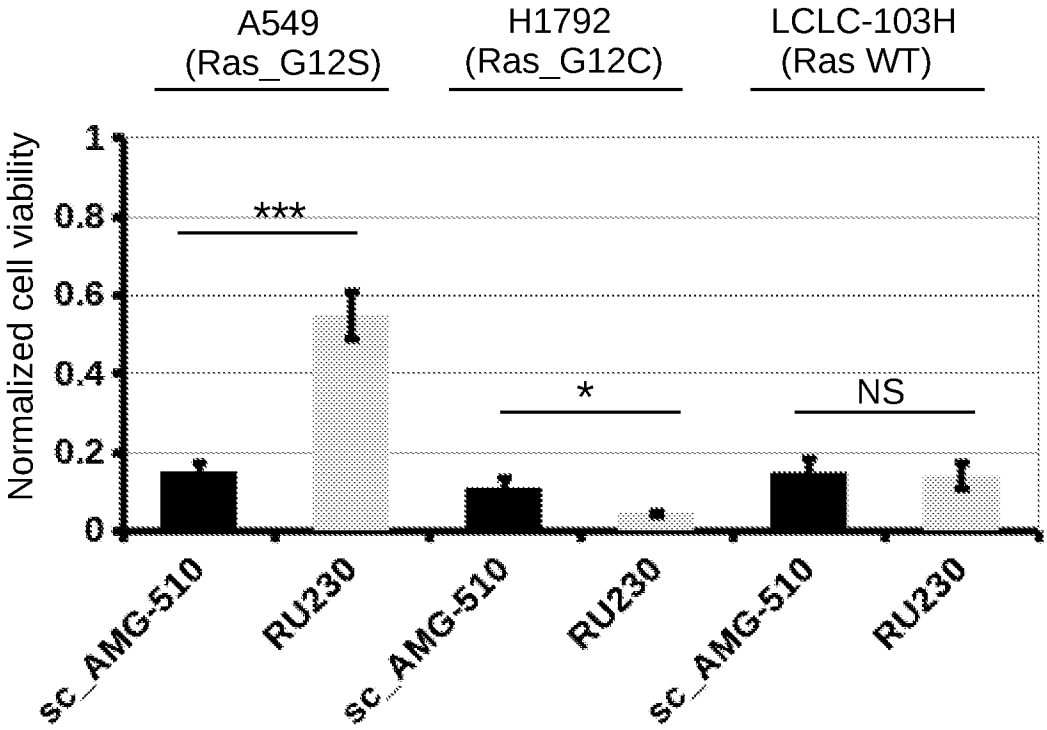
FIGURE 20-3



sc_AMG-510



RU230



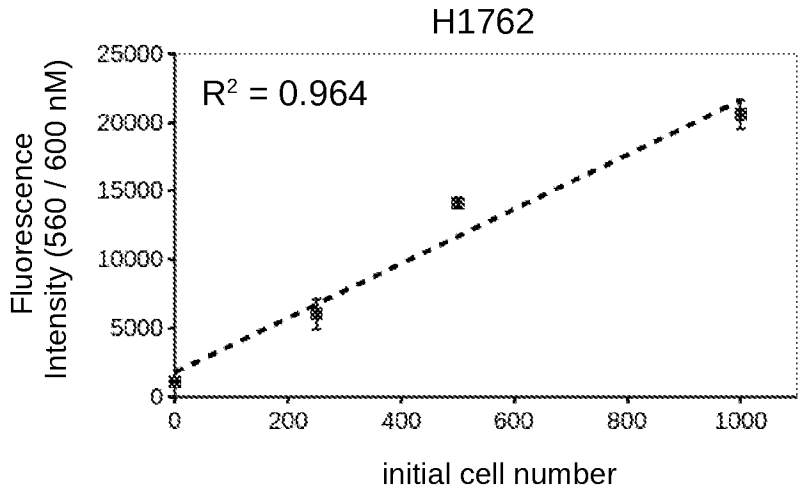


FIGURE 21