

PRODRUGS OF GLUTAMINE ANALOGS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No.

- 5 62/199,566, filed July 31, 2015, which is incorporated herein by reference in its entirety.

BACKGROUND

The prodrug approach is a well-established strategy to improve
10 physicochemical, biopharmaceutic and pharmacokinetic properties of potential drug molecules. Approximately 5-7% of drugs approved worldwide are prodrugs with annual sales in 2013 of \$11.2 billion. Most prodrugs are simple chemical derivatives of the original molecule. Ester prodrugs, the most common prodrugs, constitute 49% of all marketed prodrugs. Reasons for the popularity of ester prodrugs include their
15 generally straight forward synthesis, their improved lipophilicity and membrane permeability, and the ubiquitousness of esterase. An example of an approach to make an ester prodrug is capping the acidic moiety (ies) with lipophilic alkyl or alkyloxymethyl esters (i.e., pivaloyloxymethyl (POM) or propyloxy-carbonyloxymethyl (POC); e.g., Enalapril, Adefovir). Another approach is to cap the
20 acidic moiety(ies) with amino acids to make amides that are recognizable by amidases/peptidases in plasma for hydrolysis or to make them substrates for transporters, such as Peptide transporter 1 (PEPT1) (e.g., Pomaglumetad methionil, Valacyclovir).

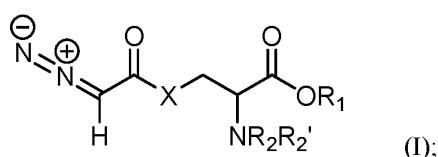
Glutamine antagonists, such as 6-Diazo-5-oxo-L-norleucine (DON), and aza-
25 serine, have been shown to exhibit broad anti-viral (Antiviral Res. 1997; 33(3):165-75; Antiviral Res. 1994; 25(3-4):269-79), anti-infective (J. Bacteriol. 1965; 89:1348-53), anti-cancer (see, e.g., Yoshioka et al., 1992; *Tokushima J. Exp. Med.* 39(1-2):69-76), anti-inflammatory, and immunosuppressive activities (Kulcsar et al., 2014; 111:16053-58; Maciolek et al., 2014; *Curr Opin Immunol.* 27:60-74; Carr et al., 2010; 30 *J Immunol.* 185:1037-1044; Colombo et al., 2010; *Proc Natl Acad Sci USA.* 107:18868-73), as well as inhibition of convulsions (*Proc R Soc Lond B Biol Sci.* 1984 Apr 24; 221(1223):145-68), multiple sclerosis (Tohoku, J. Exp. Med. 2009; 217(2):87-92), epilepsy, and viral encephalitis (J. Neurovirol. 2015 Apr; 21(2):159-73. doi: 10.1007/s13365-015-0314-6), in many published preclinical and several

clinical studies. However, the occurrence of severe toxicity (e.g., dose limiting GI toxicities, such as oral mucositis, gastric bleeding, nausea and vomiting, abdominal pain, leukopenia, thrombocytopenia, and the like) when administering such glutamine antagonists at therapeutic dose levels has hampered their clinical development.

- 5 Prior attempts to mitigate the severe toxicity associated with glutamine antagonists, such as DON, have been unsuccessful. For example, dividing daily dosing and administering of DON every four to six hours apparently doubled DON's toxicity potential (MgGill, et al., 1957). In another example, development of a treatment involving DON dosed with glutaminase to decrease plasma glutamine so
10 that the DON dose could be reduced was halted after publication of a clinical trial.

SUMMARY

- The presently disclosed subject matter provides prodrugs of glutamine antagonists, and pharmaceutically acceptable salts and esters thereof. In some
15 aspects, the presently disclosed subject matter provides a prodrug of a glutamine antagonist, or a pharmaceutically acceptable salt or ester thereof, the prodrug having a structure of formula (I):



- wherein: X is selected from the group consisting of a bond, $-O-$, and $-(CH_2)_n-$
20 , wherein n is an integer selected from the group consisting of 1, 2, 3, 4, 5, 6, 7, and 8;
 R_1 is selected from the group consisting of H and a first prodrug-forming moiety capable of forming a salt or an ester; and R_2 is H or a second prodrug-forming moiety capable of forming an amide linkage, a carbamate linkage, a phosphoramidate linkage or a phosphorodiamidate linkage with the nitrogen adjacent to R_2 ; R_2' is selected from
25 the group consisting of H, C_1 - C_6 alkyl, substituted C_1 - C_6 alkyl, or R_2 and R_2' together form a ring structure comprising $-C(=O)-G-C(=O)-$, wherein G is selected from the group consisting of C_1 - C_8 alkylene, C_1 - C_8 heteroalkylene, C_5 - C_8 cycloalkylene, C_6 - C_{12} arylene, C_5 - C_{14} heteroarylene, bivalent C_4 - C_{10} heterocycle, each of which can be optionally substituted; or R_1 and R_2' together form a 4-to 6-membered heterocyclic ring
30 comprising the oxygen atom adjacent to R_1 and the nitrogen atom adjacent to R_2' ;

provided that the compound has at least one prodrug-forming moiety selected from the group consisting of the first and the second prodrug-forming moieties.

In other aspects, the presently disclosed subject matter provides a pharmaceutical composition comprising a compound of formula (I), and a
5 pharmaceutically acceptable carrier, diluent, or excipient.

In certain aspects, the presently disclosed subject matter provides a method for treating a disease or a condition, the method comprising administering to a subject in need of treatment thereof, a compound of formula (I), or a pharmaceutical composition thereof, in an amount effective for treating the disease or condition. In
10 still other aspects, the presently disclosed subject matter provides the use of a compound of formula (I), or a pharmaceutical composition thereof, for treating a disease or condition. In some embodiments, the disease or condition is selected from the group consisting of an infection, cancer, an autoimmune disease, an inflammatory disease, and a neurodegenerative or neurological disease.

15 In yet another aspect, the presently disclosed subject matter provides a compound of formula (I), or a pharmaceutically composition thereof, for use as a medicament.

In yet another aspect, the presently disclosed subject matter provides a compound of formula (I), or a pharmaceutically composition thereof, for use in the
20 treatment of a disease or condition, preferably the disease or condition is selected from the group consisting of an infection, cancer, an autoimmune disease, an inflammatory disease, and a neurodegenerative or neurological disease.

In yet another aspect, the presently disclosed subject matter provides a compound of formula (I) or a pharmaceutically composition thereof, for use in the
25 treatment of the excess and/or aberrant glutamine activity.

Certain aspects of the presently disclosed subject matter having been stated hereinabove, which are addressed in whole or in part by the presently disclosed subject matter, other aspects will become evident as the description proceeds when taken in connection with the accompanying Examples and Figures as best described
30 herein below.

BRIEF DESCRIPTION OF THE FIGURES

Having thus described the presently disclosed subject matter in general terms, reference will now be made to the accompanying Figures, which are not necessarily drawn to scale, and wherein:

5 FIG. 1 shows exemplary amino (R) and carboxy (R') modifications made to DON in an attempt to synthesize various slow release DON prodrugs of the presently disclosed subject matter;

FIG. 2A and FIG. 2B demonstrate chemical stability challenges encountered when attempting to synthesize DON prodrugs, including stability issues presented by
10 DON esters (FIG. 2A), and DON compounds possessing a free terminal carboxylate (FIG. 2B);

FIG. 3A, FIG. 3B, FIG. 3C and FIG. 3D demonstrate the failed chemistry and poor pharmacokinetics of certain attempts to design DON prodrugs. FIG. 3A, FIG. 3B, FIG. 3C and FIG. 3D show that most DON prodrugs having a free
15 carboxylate group exhibited negligible exposure compared to DON (except for **26**, which showed some release; FIG. 3A) when administered orally in mice, including **29** (FIG. 3B), **23** (FIG. 3C) and **30** (FIG. 3D), which showed little DON release in mouse plasma upon oral administration. Note that release of DON prodrug **LTP073** was below the limit of quantitation in all mouse plasma samples at 30 minutes and 5 hours
20 after dosing (not shown);

FIG. 4A, FIG. 4B, FIG. 4C and FIG. 4D show percentages of DON released in plasma after 0.5 h and 5 h following administration of exemplary DON-releasing prodrugs of the presently disclosed subject matter as compared to DON. FIG. 4A and FIG. 4B show the results of a pharmacokinetic study in mice showing plasma DON
25 concentrations following administration of DON (1.6 mg/kg p.o.) and DON prodrugs dosed at 1.6 mg/kg equivalent of DON, such as **22** (FIG. 4A; 2.5 mg/kg p.o.) and **25** (FIG. 4B; 2.9 mg/kg p.o.). Interestingly, **22** showed lesser release at 0.5 h (65% of DON), however, the levels were similar at 5 h (almost 100%) to that of DON. FIG. 4C and FIG. 4D show the results of a pharmacokinetic study in mice showing plasma
30 DON concentrations following administration of DON (1.6 mg/kg i.p.) and DON prodrugs **17** (FIG. 4C; 3.5 mg/kg p.o.) and **6** (FIG. 4D; 3.7 mg/kg p.o.);

FIG. 5 shows the stability of DON prodrug **25** in phosphate-buffered saline (PBS) and simulated gastric fluid (SGF) after 30 min and 60 min;

FIG. 6 shows the results of an *in vitro* metabolic stability screen of DON prodrug **25** in mouse, monkey, pig, and human plasma after 30 min and 60 min. **25** was found to be unstable in plasma in all tested species as both the ethyl ester and leucine ester were hydrolysed by plasma esterases;

5 FIG. 7 shows the generic structure of certain cell targeting DON prodrugs that comprise an isopropyl ester carboxy modification;

FIG. 8 shows the results of an *in vitro* metabolic stability screen of cell targeted phosphamide DON prodrug **4** in mouse, monkey, pig, and human plasma after 30 min and 60 min. **4** was found to be unstable in mouse plasma, but stable in
10 the plasma of all other tested species;

FIG. 9 shows the results of an *in vitro* metabolic stability screen of DON prodrug **7** in mouse, monkey, pig, and human plasma after 30 min and 60 min. **7** was found to be unstable in mouse plasma, but stable in plasma of all other tested species;

FIG. 10 shows the results of an *in vitro* metabolic stability screen of DON
15 prodrug **9** in dog, mouse, monkey, pig, and human plasma after 30 min and 60 min. **9** was found to be unstable in plasma off all tested species. Metabolic identification suggested hydrolysis of leucine, but stable isopropyl ester for specific release inside lymphoid cells;

FIG. 11A, FIG. 11B, FIG. 11C, FIG. 11D, FIG. 11E and FIG. 11F
20 demonstrate the results of an *ex vivo* study comparing the accumulation of DON and DON prodrugs in plasma, blood cells, red blood cells (RBC), and peripheral blood mononuclear cells (PBMC) after 30 minutes of incubation of 20 μ M of DON (FIG. 11A) and DON prodrugs **4** (FIG. 11B), **7** (FIG. 11C), **9** (FIG. 11D), **13** (FIG. 11E) and **14** (FIG. 11F) in whole human blood;

25 FIG. 12A, FIG. 12B, FIG. 12C, and FIG. 12D demonstrate the results of *ex vivo* studies comparing the accumulation of DON and DON prodrugs in Cynomolgous monkey plasma, blood cells, RBC and PBMC after 30 minutes of incubation of 20 μ M DON (FIG. 12A) and DON prodrugs, including 20 μ M **4** (FIG. 12B), **7** (FIG. 12C) and **9** (FIG. 12C), in monkey whole blood samples;

30 FIG. 13A, FIG. 13B, and FIG. 13C demonstrate the results of *ex vivo* studies comparing the accumulation of DON and DON prodrugs in pig plasma, blood cells, RBC and PBMC after 30 minutes of incubation of 20 μ M DON (FIG. 13A) and DON prodrugs, including 20 μ M **7** (FIG. 13B) and **9** (FIG. 13C), in pig whole blood samples;

FIG. 14 shows that daily high dose treatment leads to weight loss in DON treated mice. No weight loss was observed in **25** treated mice;

FIG. 15 shows that daily high dose treatment leads to pan-cytopenia in DON treated mice. Mice treated with **25** exhibited some leukopenia;

5 FIG. 16 shows that renal and liver function in mice treated with either high dose DON or **25** was unaffected;

FIG. 17A, AND FIG. 17B show that **25** and DON treatment cure lymphoma; DON mice die from treatment while **25** is well tolerated;

FIG. 18A, FIG 18B, and FIG 18C shows that compound **25** (FIG. 18A)
10 reduces C_{max} ; (FIG. 18B) enhances tumor to gut ratio; and (FIG. 18C) causes less GI toxicity in mice vs DON at equimolar doses in mice. Ova vaccinia virus infection was given followed by daily treatment with vehicle (Veh), DON (0.8 mg/kg), and **25** (0.8 mg/kg equivalent) for 5 days. At 1 hr after last dose, animals were sacrificed, small intestines removed, perfused with DPBS pH 7.4, and rolled longitudinally using
15 the “swiss role” technique, and fixative for paraffin wax embedding. Upper panels show hematoxylin and eosin (H&E) stained sections of jejunum-ileum at 4X magnification; lower panel shows same sections at 10X magnification. DON caused significant inflammation, consisting of multifocal crypt distortion (left circle on the bottom left picture) and dilatation with loss of columnar epithelial morphology
20 (right circle on the bottom left picture). DON prodrug looked more similar to vehicle-treated mice, with slightly hypercellular crypts, but no distortion (left circle on the bottom right picture) and normal columnar morphology (right circle on the bottom right picture);

FIG. 19A and FIG. 19B show that no bone marrow suppression was observed
25 (FIG. 19A) following **25** at two 14-day effective dosing schemes; (FIG. 19B) employing long term **25** dosing schemes (48 days);

FIG. 20 shows exemplary structures of DON and DON-based prodrugs;

FIG. 21A and FIG. 21B show that DON (**1**) inhibits glutamine metabolism and glioblastoma multiform (GBM) tumor growth in vivo. FIG. 21A shows compound
30 DON (**1**) (0.8 mg/kg, i.p.) inhibited glutamine metabolism as evidenced by increased endogenous glutamine concentrations in flank GBM tumors 2 hours post-administration relative to vehicle-treated controls; * $p < 0.05$. FIG. 21B shows in efficacy studies, compared to Day 0 baseline, vehicle-treated mice exhibited significant growth of flank GBM tumors during the course of the experiment. By

contrast, systemic administration of **1** (0.8 mg/kg, i.p, q.d. days 1-6) caused a dramatic reduction in tumor size; *** $p < 0.001$, **** $p < 0.0001$;

FIG. 22A, FIG. 22B, and FIG. 22C show vivo brain and plasma pharmacokinetics of compound DON (**1**) following oral administration of DON (**1**) and **14** in mice. **1** and **14** were dosed in mice at 0.8 mg/kg equivalent, via oral gavage and plasma and brain concentrations of compound **1** were evaluated via LC/MS. Oral administration of compound **1** and **14** exhibited similar plasma and brain pharmacokinetic profiles due to complete and rapid metabolism of **14** to **1** in mouse plasma;

FIG. 23A, FIG. 23B, and FIG. 23C show in vivo pharmacokinetics of DON following intravenous (i.v.) administration of DON (**1**) and **14** in monkey plasma and cerebrospinal fluid (CSF). **1** and **14** were dosed in two pigtail macaques at 1.6 mg/kg equivalent of **1** via i.v. administration and plasma (0.25-6h) and CSF (30 min) concentrations of DON were evaluated via LC/MS. Relative to **1**, **14** delivered substantially lower DON plasma concentration. The reverse was observed in CSF, where **14** delivered significantly higher DON CSF concentrations, achieving a 10-fold enhanced CSF to plasma ratio at 30 minute post dose;

FIG. 24 shows species specific plasma stability of **14**; **14** is stable in plasma of human, pig, dog and monkeys, but rapidly metabolized in mice;

FIG. 25 shows exemplary structures of DON and DON-based prodrugs **25**, **9**, **38** and **60**; different N-amino acid promoeities (e.g., leucine, tryptophan) provide differential plasma and microsomal stability;

FIG. 26A, FIG. 26B, FIG. 26C, and FIG. 26D show in vitro plasma stability of DON prodrugs **9**, **25**, **38** and **60**. Metabolism occurs via removal of the N-protecting group; both ethyl and isopropyl esters are stable in plasma of pigs and humans;

FIG. 27A, FIG. 27B, FIG. 27C, and FIG. 27D shows in vitro liver microsomal stability of DON prodrugs **9**, **25**, **38** and **60**; all prodrugs showed moderate-high stability in human and pig microsomes;

FIG. 28A, FIG. 28B, FIG. 28C, FIG. 28D, FIG. 28E, FIG. 28F, FIG. 28G, FIG. 28H, FIG. 28I, and FIG. 28J show ex-vivo studies in whole human and pig blood of **9**, **25**, **38** and **60**; DON prodrugs selectively deliver DON to PBMCs in both humans and pigs vs plasma; compared to DON, the PBMC/plasma ratio was enhanced 10-100+ fold;

FIG. 29A FIG. 29B, FIG. 29C, FIG. 29D and FIG. 29E show pig in vivo studies with DON prodrugs of **9**, **25**, **38** and **60**; DON prodrugs selectively deliver DON to PBMCs vs plasma; compared to DON, the PBMC/plasma ratio was enhanced 6- to 10-fold;

5 FIG. 30A, FIG. 30B, and FIG. 30C show plasma stability of compound Methyl-POM **14** and its derivatives;

FIG. 31 shows exemplary structures of N-acylalkoxy DON-based prodrug analogs for intracellular targeting and brain penetration; the addition of steric bulk to the "bridge" might result in a slower hydrolysis;

10 FIG. 32A, FIG. 32B, and FIG. 32C show pig i.v. studies with DON prodrugs: in vivo in pigs DON prodrugs selectively deliver DON to PBMCs vs DON;

FIG. 33 shows PBMC to plasma AUC_(0-t) ratio comparison DON prodrugs achieve >6 fold better ratio (based on AUC_(0-t)) vs DON;

15 FIG. 34A and FIG. 34B show monkey IV studies with DON prodrug **14** shows lower plasma but higher CSF concentrations achieving ~6-7 fold >CSF/plasma ratio vs DON;

FIG. 35 shows CSF to plasma ratio at 1h after i.v. infusion with compounds **1**, **9**, **14a**, **25** and **60**;

20 FIG. 36 show clinical findings for DON (**1**) and **38** over 5 days observation period DON-treated pigs exhibited more adverse clinical signs versus **38**; DON (1.6 mg/kg) and **38** (1.5 mg/kg) doses were chosen to provide equivalent PBMC exposure; drugs were dosed i.v. for 2h/day for 5 days (n=2 pigs); close clinical monitoring was performed to evaluate drug toxicity (e.g. lethargy, anorexia, diarrhea, GI-bleeding, etc); after 5 days of dosing, pigs were euthanized and tissues were harvested for PK and histopathology;

FIG. 37A, FIG. 37B, FIG. 37C, and FIG. 37D shows that macroscopically, DON-treated pigs exhibited more severe stomach toxicity versus **38**; histopathologic toxicity scoring for gastro-intestinal and other tissues is pending; and

30 FIG. 38 shows that at equivalent PBMC exposures, **38** administration delivers less DON to GI tissues versus DON administration; DON administration appeared to cause more adverse clinical effects versus **38**.

The patent or application file contains at least one figure executed in color. Copies of this patent or patent application publication with color figures will be provided by the Office upon request and payment of the necessary fee.

DETAILED DESCRIPTION

The presently disclosed subject matter now will be described more fully hereinafter with reference to the accompanying Figures, in which some, but not all embodiments of the inventions are shown. Like numbers refer to like elements throughout. The presently disclosed subject matter may be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will satisfy applicable legal requirements. Indeed, many modifications and other embodiments of the presently disclosed subject matter set forth herein will come to mind to one skilled in the art to which the presently disclosed subject matter pertains having the benefit of the teachings presented in the foregoing descriptions and the associated Figures. Therefore, it is to be understood that the presently disclosed subject matter is not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims.

I. Prodrugs of Glutamine Antagonists

DON is an antibiotic that was isolated from *Streptomyces* in 1953. DON is a diazo analog of L-glutamine, which interferes in various reactions in the synthesis of nucleic acids and proteins in which L-glutamine donates nitrogen, blocks various glutamine recognizing enzymes, such as glutaminase, modulates brain glutamate levels, and is involved in energy metabolism, amongst others.

One strategy to improve the therapeutic index of DON for various treatment regimes, including GBM therapy, would be to increase its brain exposure while limiting its systemic exposure and thus toxicity (Upadhyay, 2014). The prodrug approach is a well-established strategy to alter the pharmacokinetic and tissue distribution of drug molecules, however synthetically this approach is challenging with DON. Given that DON's labile diazo group is critical to its glutamine antagonizing activity, the addition of promoeities must be performed under mild conditions to preserve the diazo ketone group.

The presently disclosed subject matter provides novel compositions of matter wherein pro-moieties have been added to glutamine antagonists, such as 6-diazo-5-oxo-norleucine (DON), and aza-serine.

The presently disclosed prodrugs of glutamine antagonists were prepared by masking the amine and/or the carboxylate functionalities to alter the pharmacokinetics of DON providing slower release kinetics and cellular targeting to enhance

tolerability. Further, the presently disclosed prodrugs, in some embodiments, selectively target the active glutamine antagonists to specific cells or provide a slower release of DON and thus decrease the toxicity of the drug molecule.

- 5 The presently disclosed subject matter demonstrates that masking both the α -amino group and the carboxy-functionality to be derivatized enhances prodrug stability and oral bioavailability. The presently disclosed prodrugs also exhibit a stability that is comparable to free DON.

Structures of representative DON prodrugs are provided in Table 1.

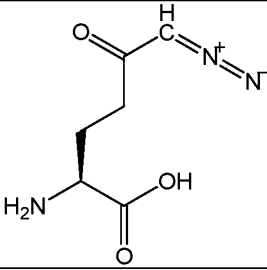
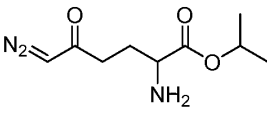
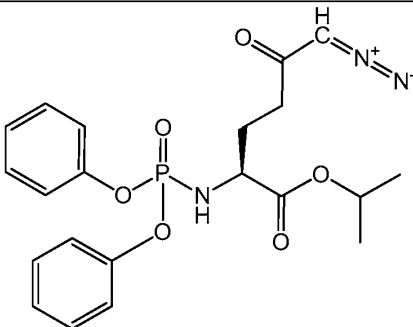
Table 1. Structures of Representative DON Prodrugs		
IOCB No./ Compound No.	Structure	MW
Compound 1 (DON)		171.15
Compound 3		213.24
Compound 4		445.41

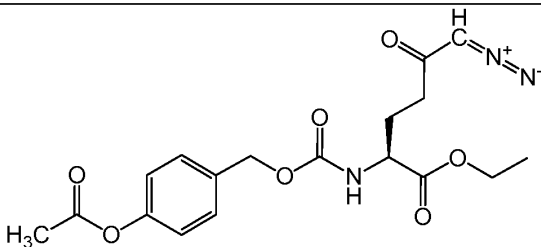
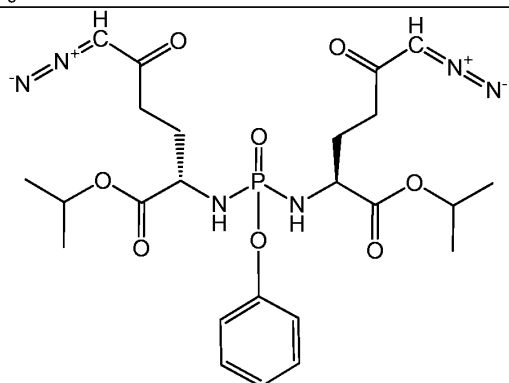
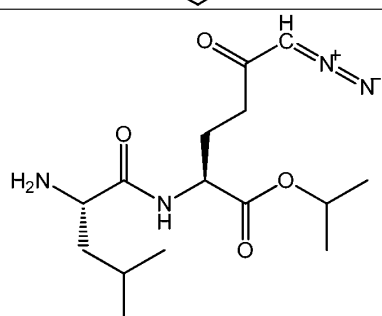
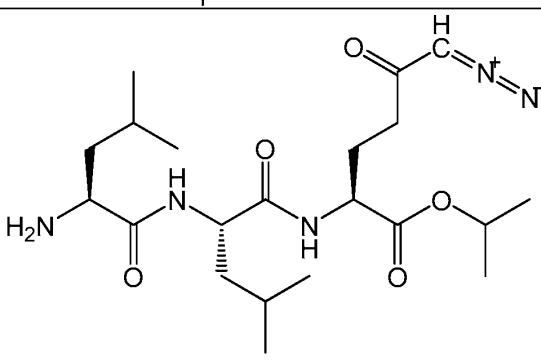
Table 1. Structures of Representative DON Prodrugs		
IOCB No./ Compound No.	Structure	MW
Compound 6		391.38
Compound 7		564.53
Compound 9		326.39
Compound 11		439.55

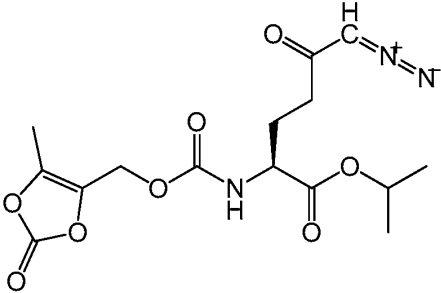
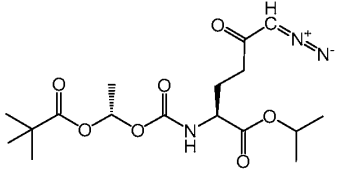
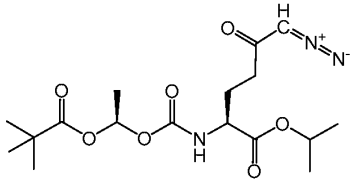
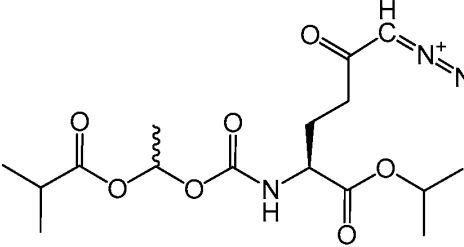
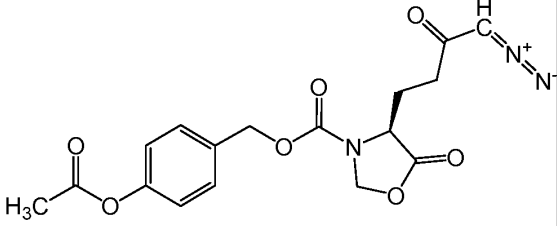
Table 1. Structures of Representative DON Prodrugs		
IOCB No./ Compound No.	Structure	MW
Compound 13		369.18
Compound 14a		385.41
Compound 14b		
Compound 15		371.39
Compound 17		375.33

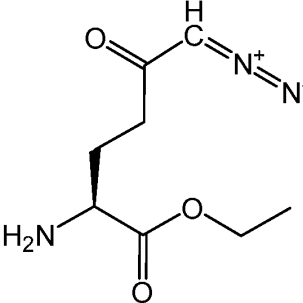
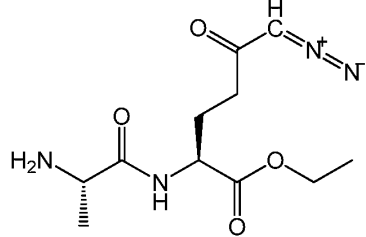
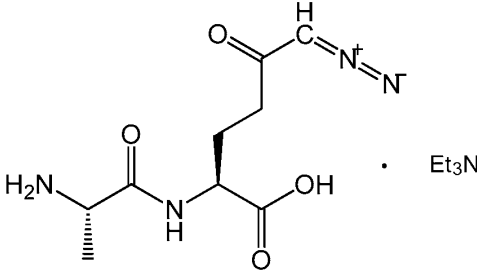
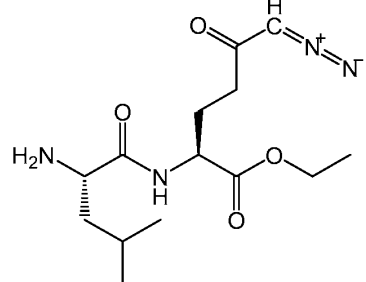
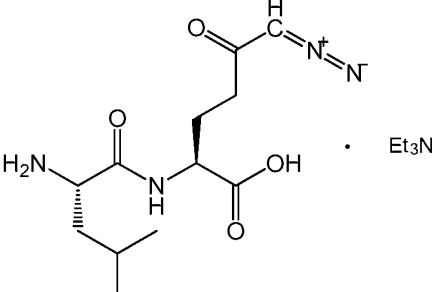
Table 1. Structures of Representative DON Prodrugs		
IOCB No./ Compound No.	Structure	MW
Compound 20		199.21
Compound 22		270.28
Compound 23		343.42
Compound 25		312.36
Compound 26		385.50

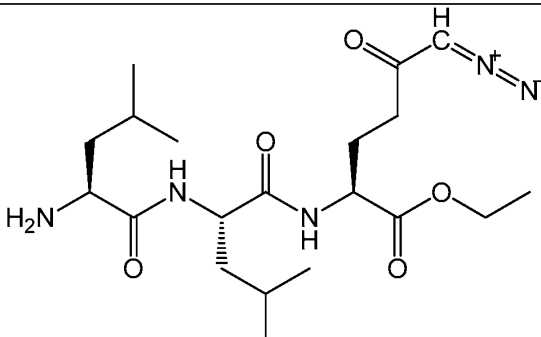
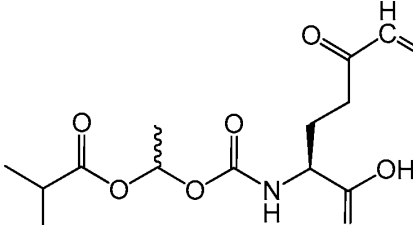
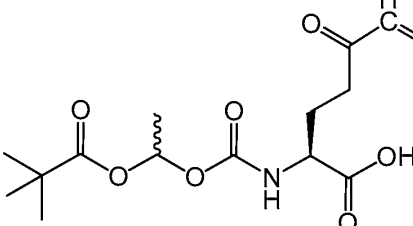
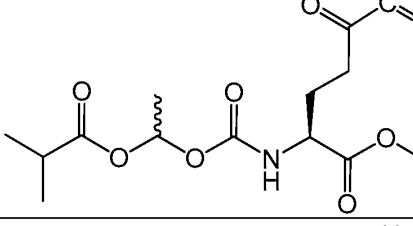
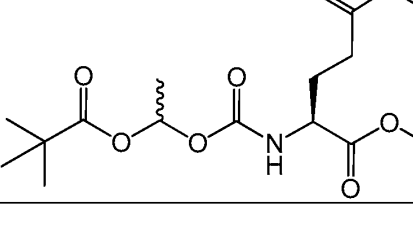
Table 1. Structures of Representative DON Prodrugs		
IOCB No./ Compound No.	Structure	MW
Compound 28		425.52
Compound 29		329.31
Compound 30		343.33
Compound 31		357.37
Compound 32		371.39

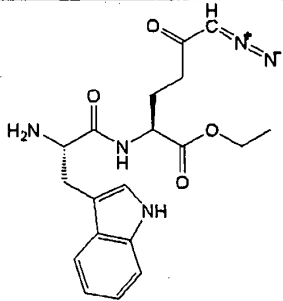
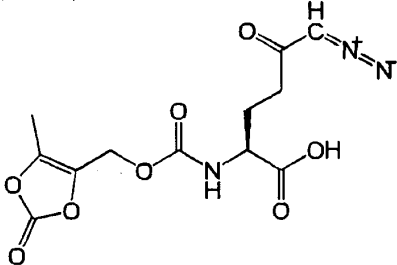
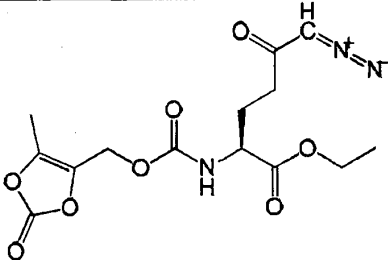
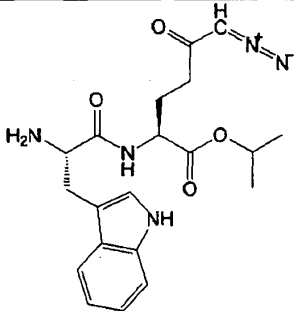
Table 1. Structures of Representative DON Prodrugs		
IOCB No./ Compound No.	Structure	MW
Compound 34		385.42
Compound 35		327.25
Compound 36		355.30
Compound 38		399.45

Table 1. Structures of Representative DON Prodrugs		
IOCB No./ Compound No.	Structure	MW
Compound 40		413.47
Compound 42		371.39
Compound 44		2.44
Compound 47		447.49
Compound 49		357.36

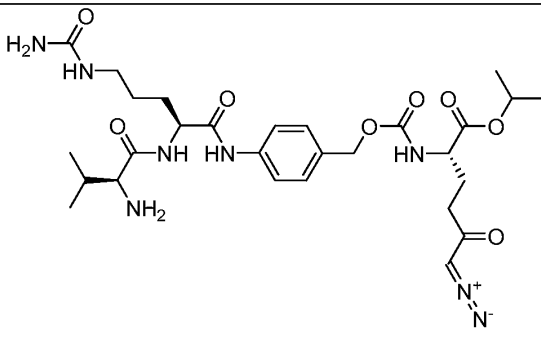
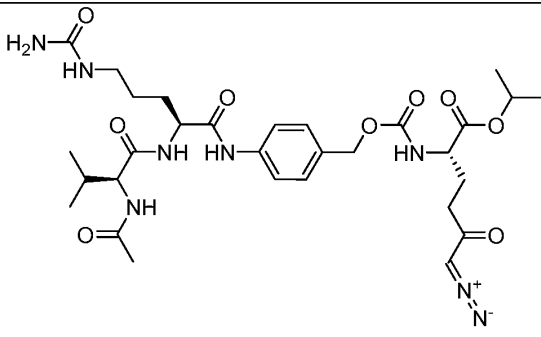
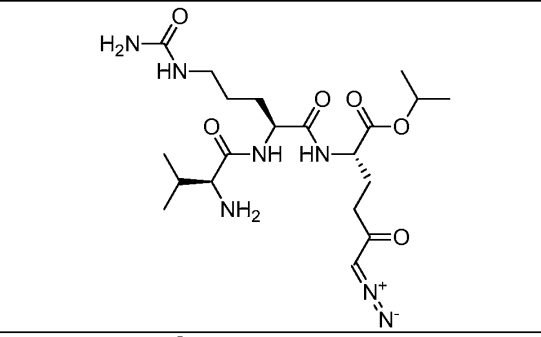
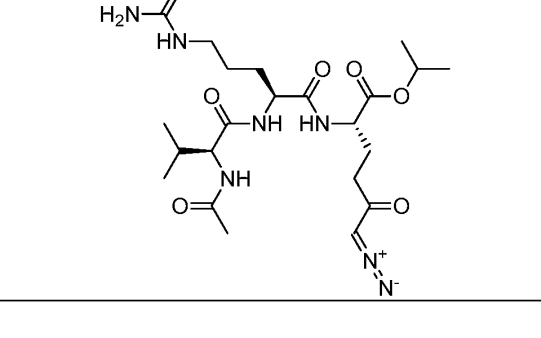
Table 1. Structures of Representative DON Prodrugs		
IOCB No./ Compound No.	Structure	MW
Compound 51		618.69
Compound 52		660.73
Compound 56		469.54
Compound 57		511.58

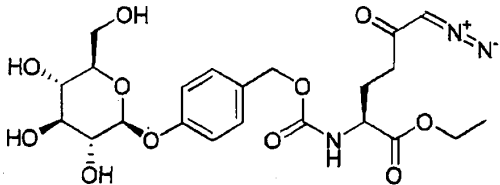
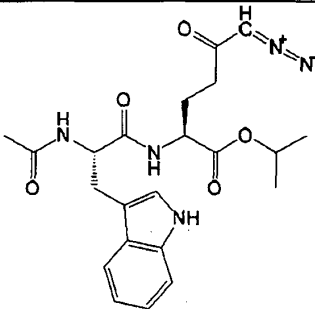
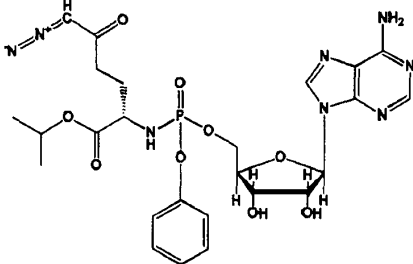
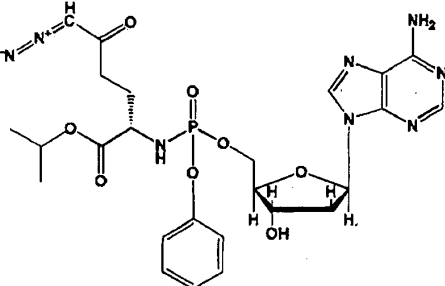
Table 1. Structures of Representative DON Prodrugs		
IOCB No./ Compound No.	Structure	MW
Compound 59		511.48
Compound 60		464.19
<u>A</u>		618.54
<u>B</u>		602.54

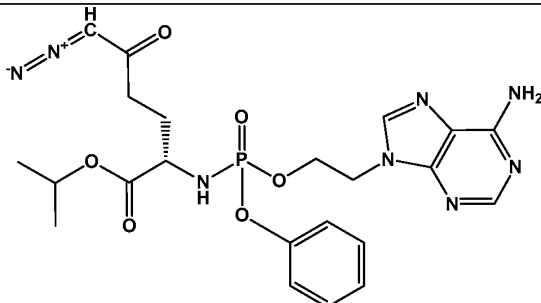
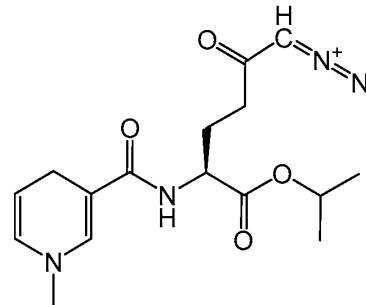
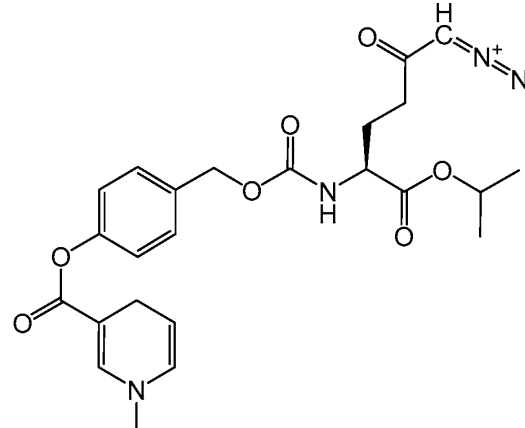
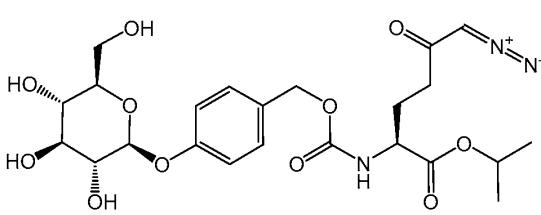
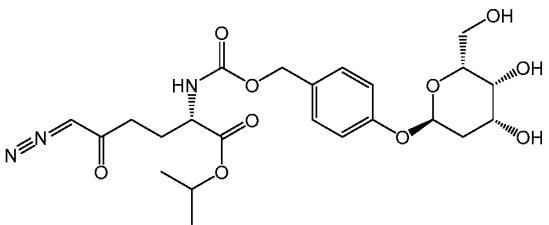
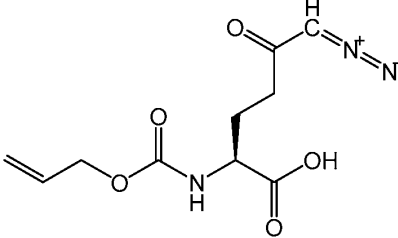
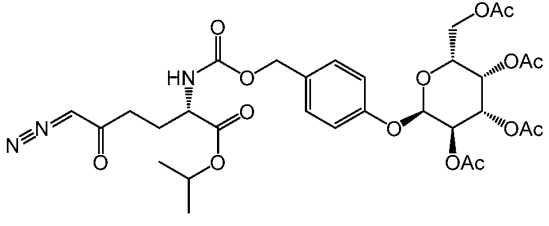
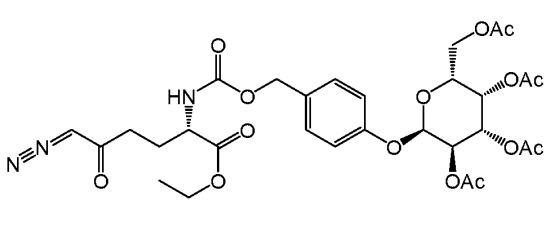
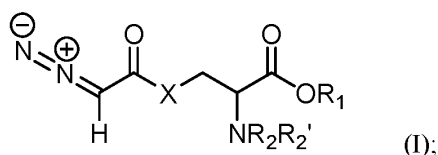
Table 1. Structures of Representative DON Prodrugs		
IOCB No./ Compound No.	Structure	MW
<u>C</u>		530.47
<u>D</u>		334.38
<u>E</u>		484.51
<u>F</u>		525.51

Table 1. Structures of Representative DON Prodrugs		
IOCB No./ Compound No.	Structure	MW
<u>G</u>		509.51
<u>LTP 073</u>		255.23
<u>JAM0351</u>		693.66
<u>JAM0359</u>		679.63

Those skilled in the art will appreciate that the representative structures of the DON prodrugs shown in Table 1 combined with the guidance disclosed herein will enable those skilled in the art to synthesize prodrugs of other glutamine analogs, such as L-DONV, aza-serine, as are exemplified in the generic structures of formula (I). In other words, it should be understood that the prodrugs of other glutamine antagonists, such as L-DONV, aza-serine, can be synthesized with the same substituents R₁, R₂ and R₂' as the DON prodrugs shown in Table 1.

Accordingly, in one aspect the presently disclosed subject matter provides a prodrug of a glutamine antagonist, or a pharmaceutically acceptable salt or ester

thereof, the prodrug having a structure of formula (I):

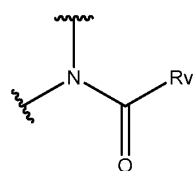


wherein: X is selected from the group consisting of a bond, $-O-$, and $-(CH_2)_n-$,

wherein n is an integer selected from the group consisting of 1, 2, 3, 4, 5, 6, 7, and 8;

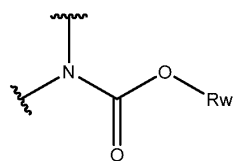
- 5 R_1 is selected from the group consisting of H and a first prodrug-forming moiety capable of forming a salt or an ester; and R_2 is H or a second prodrug-forming moiety capable of forming an amide linkage, a carbamate linkage, a phosphoramidate linkage or a phosphorodiamidate linkage with the nitrogen adjacent to R_2 ; R_2' is selected from the group consisting of H, C_1 - C_6 alkyl, substituted C_1 - C_6 alkyl, or R_2 and R_2' together form a ring structure comprising $-C(=O)-G-C(=O)-$, wherein G is selected from the group consisting of C_1 - C_8 alkylene, C_1 - C_8 heteroalkylene, C_5 - C_8 cycloalkylene, C_6 - C_{12} arylene, C_5 - C_{14} heteroarylene, bivalent C_4 - C_{10} heterocycle, each of which can be optionally substituted; or R_1 and R_2' together form a 4-to 6-membered heterocyclic ring comprising the oxygen atom adjacent to R_1 and the nitrogen atom adjacent to R_2' ; provided that the compound has at least one prodrug-forming moiety selected from the group consisting of the first and the second prodrug-forming moieties.

As used herein, the term “amide linkage” comprises a structure represented by the formula:



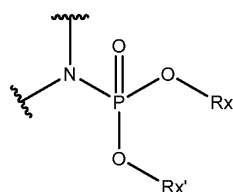
- 20 , wherein R_v is selected from the group consisting of alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, aryl, substituted aryl, aralkyl, substituted aralkyl, heterocyclyl, substituted heterocyclyl, alkenyl, substituted alkenyl, cycloalkenyl, substituted cycloalkenyl, alkylamine, substituted alkylamine, heteroaryl, and substituted heteroaryl.

- 25 As used herein, the term “carbamate linkage” comprises a structure represented by the formula:



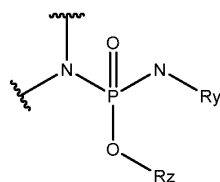
, wherein R_w is selected from the group consisting of alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, aryl, substituted aryl, aralkyl, substituted aralkyl, heterocyclyl, substituted heterocyclyl, alkenyl, substituted alkenyl, cycloalkenyl, substituted cycloalkenyl, alkylamine, substituted alkylamine, heteroaryl, and substituted heteroaryl.

As used herein, the term “phosphoramidate linkage” comprises a structure represented by the formula:

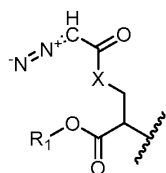


, wherein R_x and $R_{x'}$ are each independently selected from the group consisting of alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, aryl, substituted aryl, aralkyl, substituted aralkyl, heterocyclyl, substituted heterocyclyl, alkenyl, substituted alkenyl, cycloalkenyl, substituted cycloalkenyl, alkylamine, substituted alkylamine, heteroaryl, and substituted heteroaryl.

As used herein, the term “phosphorodiamidate linkage” comprises a structure represented by the formula:



, wherein R_y and R_z are each independently selected from the group consisting of H, alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, heterocyclyl, substituted heterocyclyl, alkenyl, substituted alkenyl, cycloalkenyl, substituted cycloalkenyl, $-(CR_3R_4)_m-Z$, $-(CR_3R_4)_m-Q-Z$, aryl, substituted aryl, alkylamine, substituted alkylamine, heteroaryl, substituted heteroaryl, and



20

In some embodiments, X is $-CH_2-$, and n is 1.

In other embodiments, X is -O-. In some embodiments, the prodrug compound has both the first prodrug-forming moiety and the second prodrug-forming moiety. In some embodiments, the glutamine analog is a glutamine antagonist, i.e., the prodrug is a prodrug of a glutamine analog that antagonizes a glutamine pathway.

- 5 Exemplary glutamine antagonists include, without limitation, 6-diazo-5-oxo-norleucine (DON), and aza-serine, and 5-diazo-4-oxo-L-norvaline (L-DONV).

In some embodiments, the presently disclosed subject matter provides a prodrug of DON. In some embodiments, the prodrug of DON has a structure of formula (I). In some embodiments, the presently disclosed subject matter provides a
10 prodrug of L-DONV. In some embodiments, the prodrug of L-DONV has a structure of formula (I). In some embodiments, the presently disclosed subject matter provides a prodrug of azaserine. In some embodiments, the prodrug of azaserine has a structure of formula (I).

In some embodiments, R₁ of formula (I) comprises a residue PRO₁ of the
15 prodrug-forming moiety, which, together with a basic moiety and the terminal hydroxyl group forms a salt.

In some embodiments, R₁ of formula (I) comprises a residue PRO₁ of the prodrug-forming moiety, which, together with an alkyl group and the oxygen of an adjoining hydroxyl group forms an ester.

20 In some embodiments, R₁ of formula (I) comprises a residue PRO₁ of the prodrug-forming moiety, which, together with an alkyl group and the nitrogen adjoining the R₂' group, forms an azlactone or an oxazolidone.

In some embodiments, R₁ of formula (I) is selected from the group consisting of H, alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, alkenyl, substituted
25 alkenyl, alkynyl, substituted alkynyl, cycloalkenyl, substituted cycloalkenyl, tri(hydrocarbyl)ammonium, and tetra(hydrocarbyl)ammonium. Preferred alkyl group, cycloalkyl group, alkenyl group, alkynyl group, and cycloalkenyl group substituents include alkyl, substituted alkyl, halo, arylamino, acyl, hydroxyl, aryloxy, alkoxy, alkylthio, arylthio, aralkyloxy, aralkylthio, carboxyl, alkoxy carbonyl, oxo, and
30 cycloalkyl.

In some embodiments, R₁ of formula (I) is not H. In some embodiments, R₁ of formula (I) is not H when R₂ and R₂' are H. In some embodiments, R₂ and R₂' of formula (I) are each H when R₁ is not H.

In some embodiments, R₁ of formula (I) is selected from the group consisting of a C₁₋₆ straight-chain alkyl, a substituted C₁₋₆ straight-chain alkyl, a C₁₋₆ branched alkyl, a substituted C₁₋₆ branched alkyl, tri(C₁-C₈-alkyl)ammonium, tetra(C₁-C₈-alkyl)ammonium, triphenylammonium, tri(hydroxy-C₁-C₈-alkyl)ammonium, and
5 tetra(hydroxy-C₁-C₈-alkyl)ammonium.

In some embodiments, R₁ of formula (I) is selected from the group consisting of methyl, ethyl, isopropyl, cyclopentyl, cyclohexyl, trimethylammonium, triethylammonium, tri(hydroxyethyl)ammonium, tripropylammonium, and tri(hydroxypropyl)ammonium. In some embodiments, R₁ of formula (I) is methyl. In
10 some embodiments, R₁ of formula (I) is ethyl. In some embodiments, R₁ of formula (I) is isopropyl.

In some embodiments, R₂ of formula (I) comprises a residue PRO₂ of the second prodrug-forming moiety, which, together with a carbonyl, oxy carbonyl, or phosphonyl group and the nitrogen of the adjoining NH, forms an amide, a carbamate,
15 phosphoramidate, or phosphorodiamidate linkage.

In some embodiments, R₂ of formula (I) comprises a moiety selected from the group consisting of an amino acid, an N-substituted amino acid, a peptide, a substituted peptide, a monocyclic ring, a substituted monocyclic ring, a bicyclic ring, a substituted bicyclic ring, a purine nucleoside, a substituted purine nucleoside, a
20 pyrimidine nucleoside, and a substituted pyrimidine nucleoside.

As used herein, the term "amino acid" includes moieties having a carboxylic acid group and an amino group. The term amino acid thus includes both natural amino acids (including proteinogenic amino acids) and non-natural amino acids. The term "natural amino acid" also includes other amino acids that can be incorporated
25 into proteins during translation (including pyrrolysine and selenocysteine). Additionally, the term "natural amino acid" also includes other amino acids, which are formed during intermediary metabolism, e.g., ornithine generated from arginine in the urea cycle. The natural amino acids are summarized in Table 2:

Table 2. Natural Amino Acids (Used For Protein Biosynthesis)		
Amino acid	3 letter code	1-letter code
Alanine	ALA	A
Cysteine	CYS	C
Aspartic Acid	ASP	D
Glutamic Acid	GLU	E
Phenylalanine	PHE	F
Glycine	GLY	G
Histidine	HIS	H
Isoleucine	ILE	I
Lysine	LYS	K
Leucine	LEU	L
Methionine	MET	M
Asparagine	ASN	N
Proline	PRO	P
Glutamine	GLN	Q
Arginine	ARG	R
Serine	SER	S
Threonine	THR	T
Valine	VAL	V
Tryptophan	TRP	W
Tyrosine	TYR	Y

The natural or non-natural amino acid may be optionally substituted. In one embodiment, the amino acid is selected from proteinogenic amino acids.

- 5 Proteinogenic amino acids include glycine, alanine, valine, leucine, isoleucine, aspartic acid, glutamic acid, serine, threonine, glutamine, asparagine, arginine, lysine, proline, phenylalanine, tyrosine, tryptophan, cysteine, methionine and histidine. The term amino acid includes alpha amino acids and beta amino acids, such as, but not limited to, beta alanine and 2-methyl beta alanine. The term amino acid also includes

certain lactam analogues of natural amino acids, such as, but not limited to, pyroglutamine. The term amino acid also includes amino acids homologues including homocitrulline, homoarginine, homoserine, homotyrosine, homoproline and homophenylalanine.

5 The terminal portion of the amino acid residue or peptide may be in the form of the free acid i.e., terminating in a —COOH group or may be in a masked (protected) form, such as in the form of a carboxylate ester or carboxamide. In certain embodiments, the amino acid or peptide residue terminates with an amino group. In an embodiment, the residue terminates with a carboxylic acid group —COOH or an
10 amino group —NH₂. In another embodiment, the residue terminates with a carboxamide group. In yet another embodiment, the residue terminates with a carboxylate ester.

As disclosed hereinabove, the term “amino acid” includes compounds having a —COOH group and an —NH₂ group. A substituted amino acid includes an amino
15 acid which has an amino group which is mono- or di-substituted. In particular embodiments, the amino group may be mono-substituted. (A proteinogenic amino acid may be substituted at another site from its amino group to form an amino acid which is a substituted proteinogenic amino acid). The term substituted amino acid thus includes N-substituted metabolites of the natural amino acids including, but not
20 limited to, N-acetyl cysteine, N-acetyl serine, and N-acetyl threonine.

For example, the term “N-substituted amino acid” includes N-alkyl amino acids (e.g., C₁₋₆ N-alkyl amino acids, such as sarcosine, N-methyl-alanine, N-methyl-glutamic acid and N-tert-butylglycine), which can include C₁₋₆ N-substituted alkyl amino acids (e.g., N-(carboxy alkyl) amino acids (e.g., N-(carboxymethyl)amino
25 acids) and N-methylcycloalkyl amino acids (e.g., N-methylcyclopropyl amino acids)); N,N-di-alkyl amino acids (e.g., N,N-di-C₁₋₆ alkyl amino acids (e.g., N,N-dimethyl amino acid)); N,N,N-tri-alkyl amino acids (e.g., N,N,N-tri-C₁₋₆ alkyl amino acids (e.g., N,N,N-trimethyl amino acid)); N-acyl amino acids (e.g., C₁₋₆ N-acyl amino acid); N-aryl amino acids (e.g., N-phenyl amino acids, such as N-phenylglycine); N-
30 amidinyl amino acids (e.g., an N-amidine amino acid, i.e., an amino acid in which an amine group is replaced by a guanidino group).

The term “amino acid” also includes amino acid alkyl esters (e.g., amino acid C₁₋₆ alkyl esters); and amino acid aryl esters (e.g., amino acid phenyl esters).

For amino acids having a hydroxy group present on the side chain, the term “amino acid” also includes O-alkyl amino acids (e.g., C₁₋₆ O-alkyl amino acid ethers); O-aryl amino acids (e.g., O-phenyl amino acid ethers); O-acyl amino acid esters; and O-carbamoyl amino acids.

5 For amino acids having a thiol group present on the side chain, the term “amino acid” also includes S-alkyl amino acids (e.g., C₁₋₆ S-alkyl amino acids, such as S-methyl methionine, which can include C₁₋₆ S-substituted alkyl amino acids and S-methylcycloalkyl amino acids (e.g., S-methylcyclopropyl amino acids)); S-acyl amino acids (e.g., a C₁₋₆ S-acyl amino acid); S-aryl amino acid (e.g., a S-phenyl amino acid);
10 a sulfoxide analogue of a sulfur-containing amino acid (e.g., methionine sulfoxide) or a sulfoxide analogue of an S-alkyl amino acid (e.g., S-methyl cystein sulfoxide) or an S-aryl amino acid.

In other words, the presently disclosed subject matter also envisages derivatives of natural amino acids, such as those mentioned above which have been
15 functionalized by simple synthetic transformations known in the art (e.g., as described in “Protective Groups in Organic Synthesis” by T W Greene and P G M Wuts, John Wiley & Sons Inc. (1999)), and references therein.

Examples of non-proteinogenic amino acids include, but are not limited to: citrulline, hydroxyproline, 4-hydroxyproline, β-hydroxyvaline, ornithine, β-amino
20 alanine, albizziin, 4-amino-phenylalanine, biphenylalanine, 4-nitro-phenylalanine, 4-fluoro-phenylalanine, 2,3,4,5,6-pentafluoro-phenylalanine, norleucine, cyclohexylalanine, α-aminoisobutyric acid, α-aminobutyric acid, α-aminoisobutyric acid, 2-aminoisobutyric acid, 2-aminoindane-2-carboxylic acid, selenomethionine, lanthionine, dehydroalanine, γ-amino butyric acid, naphthylalanine, aminohexanoic
25 acid, pipecolic acid, 2,3-diaminopropionic acid, tetrahydroisoquinoline-3-carboxylic acid, tert-leucine, tert-butylalanine, cyclopropylglycine, cyclohexylglycine, 4-aminopiperidine-4-carboxylic acid, diethylglycine, dipropylglycine and derivatives thereof wherein the amine nitrogen has been mono- or di-alkylated.

The term “peptide” refers to an amino acid chain consisting of 2 to 9 amino
30 acids, unless otherwise specified. In preferred embodiments, the peptide used in the present invention is 2 or 3 amino acids in length. In one embodiment, a peptide can be a branched peptide. In this embodiment, at least one amino acid side chain in the peptide is bound to another amino acid (either through one of the termini or the side chain).

The term “N-substituted peptide” refers to an amino acid chain consisting of 2 to 9 amino acids in which one or more NH groups are substituted, e.g., by a substituent described elsewhere herein in relation to substituted amino groups.

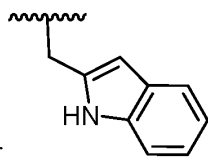
Optionally, the N-substituted peptide has its N-terminal amino group substituted and, in one embodiment, the amide linkages are unsubstituted.

In one embodiment, an amino acid side chain is bound to another amino acid. In a further embodiment, side chain is bound to the amino acid via the amino acid's N-terminus, C-terminus, or side chain.

Examples of natural amino acid sidechains include hydrogen (glycine), methyl (alanine), isopropyl (valine), sec-butyl (isoleucine), $-\text{CH}_2\text{CH}(\text{CH}_3)_2$ (leucine), benzyl (phenylalanine), p-hydroxybenzyl (tyrosine), $-\text{CH}_2\text{OH}$ (serine), $-\text{CH}(\text{OH})\text{CH}_3$ (threonine), $-\text{CH}_2$ -3-indoyl (tryptophan), $-\text{CH}_2\text{COOH}$ (aspartic acid), $-\text{CH}_2\text{CH}_2\text{COOH}$ (glutamic acid), $-\text{CH}_2\text{C}(\text{O})\text{NH}_2$ (asparagine), $-\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{NH}_2$ (glutamine), $-\text{CH}_2\text{SH}$, (cysteine), $-\text{CH}_2\text{CH}_2\text{SCH}_3$ (methionine), $-(\text{CH}_2)_4\text{NH}_2$ (lysine), $-(\text{CH}_2)_3\text{NHC}(=\text{NH})\text{NH}_2$ (arginine) and $-\text{CH}_2$ -3-imidazolyl (histidine).

Exemplary monocyclic rings and bicyclic rings include, without limitation, benzene, pyrimidines, and purines, and more generally aryl and heteroaryl rings. Exemplary heteroaryls include, but are not limited to, pyridyl, pyrimidinyl, pyrazinyl, furanyl, thienyl, pyrazolyl, oxazolyl, thiazolyl, isothiazolyl, isoxazolyl, pyrrolyl, imidazolyl, indolyl, indolinolyl, and imidazopyridazinyl. Aryls include phenyl (C_6), benzyl, naphthyl (C_{10}), and biphenyl (C_{12}). Exemplary pyrimidines include, without limitation, cytosine, thymine, and uracil. Exemplary purines include, without limitation, purine, adenine, N-substituted adenine, guanine, hypoxanthine, xanthine, theobromine, caffeine, uric acid, and isoguanine. Exemplary purine nucleosides include, without limitation, adenine and guanine.

In some embodiments, R_2 of formula (I) is selected from the group consisting of H, alkyl, $-\text{C}(=\text{O})-\text{Ar}$, $-\text{C}(=\text{O})-\text{Y}-(\text{CR}_3\text{R}_4)_m-\text{Ar}$, $-\text{C}(=\text{O})-\text{Y}-(\text{CR}_3\text{R}_4)_m-\text{NR}_5\text{R}_6$, $-\text{P}(=\text{O})(\text{OR}_7)_n(\text{NHR}_9)_o$, $-\text{C}(=\text{O})-\text{Y}-(\text{CR}_3\text{R}_4)_m-\text{Ar}-\text{O}-\text{C}(=\text{O})-\text{R}_8$, $-\text{C}(=\text{O})-\text{Y}-(\text{CR}_3\text{R}_4)_m-\text{Ar}-\text{O}-\text{R}_8$, $-\text{C}(=\text{O})-\text{O}-(\text{CR}_3\text{R}_4)_m-\text{O}-\text{C}(=\text{O})-\text{R}_{10}$, $-\text{C}(=\text{O})-\text{O}-\text{R}_9$, $-\text{C}(=\text{O})-\text{Y}-(\text{CR}_3\text{R}_4)_m-\text{Ar}-\text{O}-\text{C}(=\text{O})-\text{Ar}$, and $-\text{C}(=\text{O})-\text{Y}-(\text{CR}_3\text{R}_4)_m-\text{Ar}-\text{NR}_5\text{R}_6$; wherein: Y is $-\text{O}-$ or a bond; m is an integer selected from the group consisting of 0, 1, 2, 3, 4, 5, 6, 7, and 8; each n and o is an integer from 0 to 2 provided that the sum of n and o is 2; R_3 and R_4 are independently H, C_1 - C_6 alkyl or substituted C_1 - C_6 alkyl, aryl or substituted aryl, -

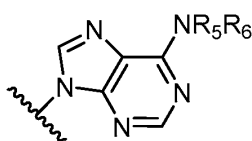


(CR₃R₄)_m-NR₅R₆, or

C(=O)-(CR₃R₄)_m, -C(=O)-(NR₅R₆), or -C(=O)-(CR₃R₄)_m-NR₅R₆; each R₅ and R₆ is independently H, alkyl, -

independently selected from the group consisting of H, alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, heterocyclyl, substituted heterocyclyl, alkenyl,

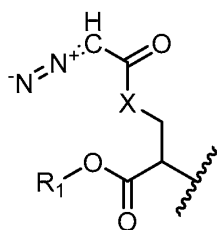
- 5 substituted alkenyl, cycloalkenyl, substituted cycloalkenyl, -(CR₃R₄)_m-Z, -(CR₃R₄)_m-Q-Z, wherein Q is a monosaccharide, aryl, substituted aryl, heteroaryl, substituted



heteroaryl, and wherein Z is

or wherein R₇ together with the oxygen atom to which it is attached forms a purine or pyrimidine nucleoside; each R₉ is independently selected from the group consisting of

- 10 H, alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, heterocyclyl, substituted heterocyclyl, alkenyl, substituted alkenyl, cycloalkenyl, substituted cycloalkenyl, -(CR₃R₄)_m-Z, aryl, substituted aryl, heteroaryl, substituted heteroaryl, and

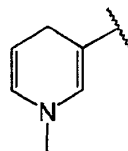
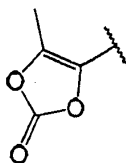


, wherein R₁ and X are as defined above, provided that R₁

is not H;

- 15 each R₈ is independently alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, monosaccharide, acylated monosaccharide, aryl, substituted aryl, heteroaryl, substituted heteroaryl; each R₁₀ is independently alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, monosaccharide, acylated monosaccharide, aryl, substituted aryl, heteroaryl, substituted heteroaryl; and Ar is aryl, substituted aryl, 20 heteroaryl, or substituted heteroaryl. It should be appreciated that in addition to substitutions on the amino group of Z, one or more substitutions R₃, R₄, R₅, and/or R₆ can be made to the 5 or 6 membered rings of Z.

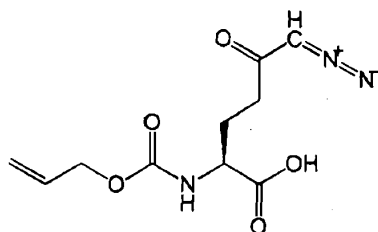
In particular embodiments Ar is



In other particular embodiments, Ar is and

In yet other particular embodiments, Ar is benzyl.

In particular embodiments, the prodrug compound of formula (I) is



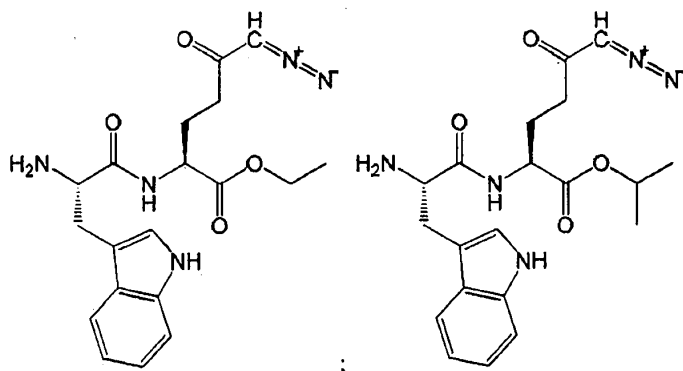
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In some embodiments, R_2 of formula (I) is $-C(=O)-Y-(CR_3R_4)_m-NR_5R_6$,

wherein: (i) Y is a bond; m is 1; R_5 and R_6 are each H; (ii) Y is a bond; m is 1; R_5 is H; R_6 is $-C(=O)-(CR_3R_4)_m$.

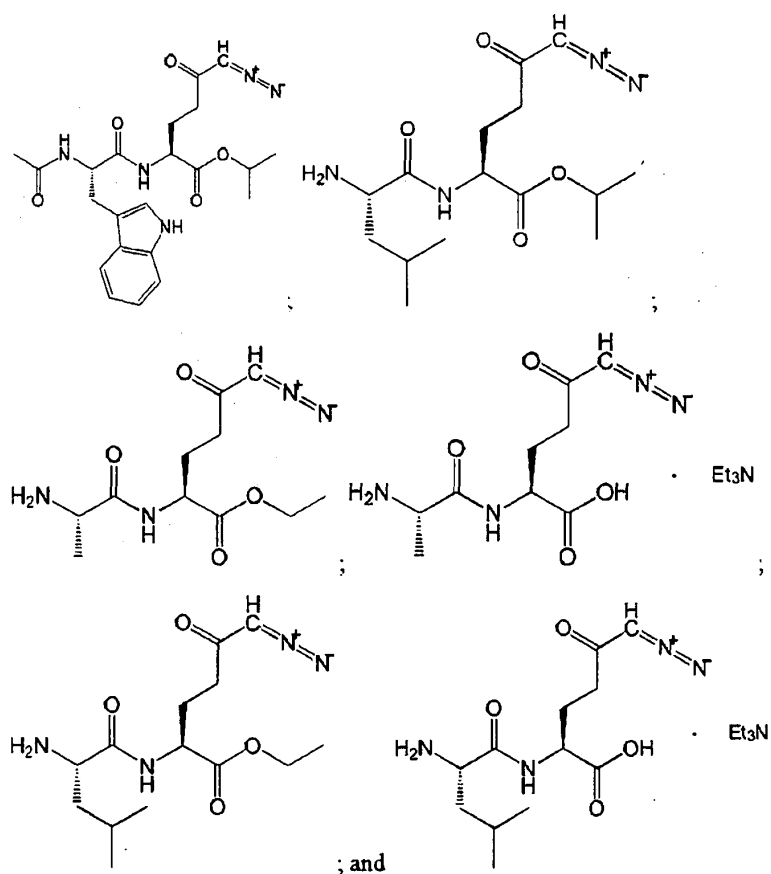
In particular embodiments, the prodrug compound of formula (I) is selected

10 from the group consisting of:



30

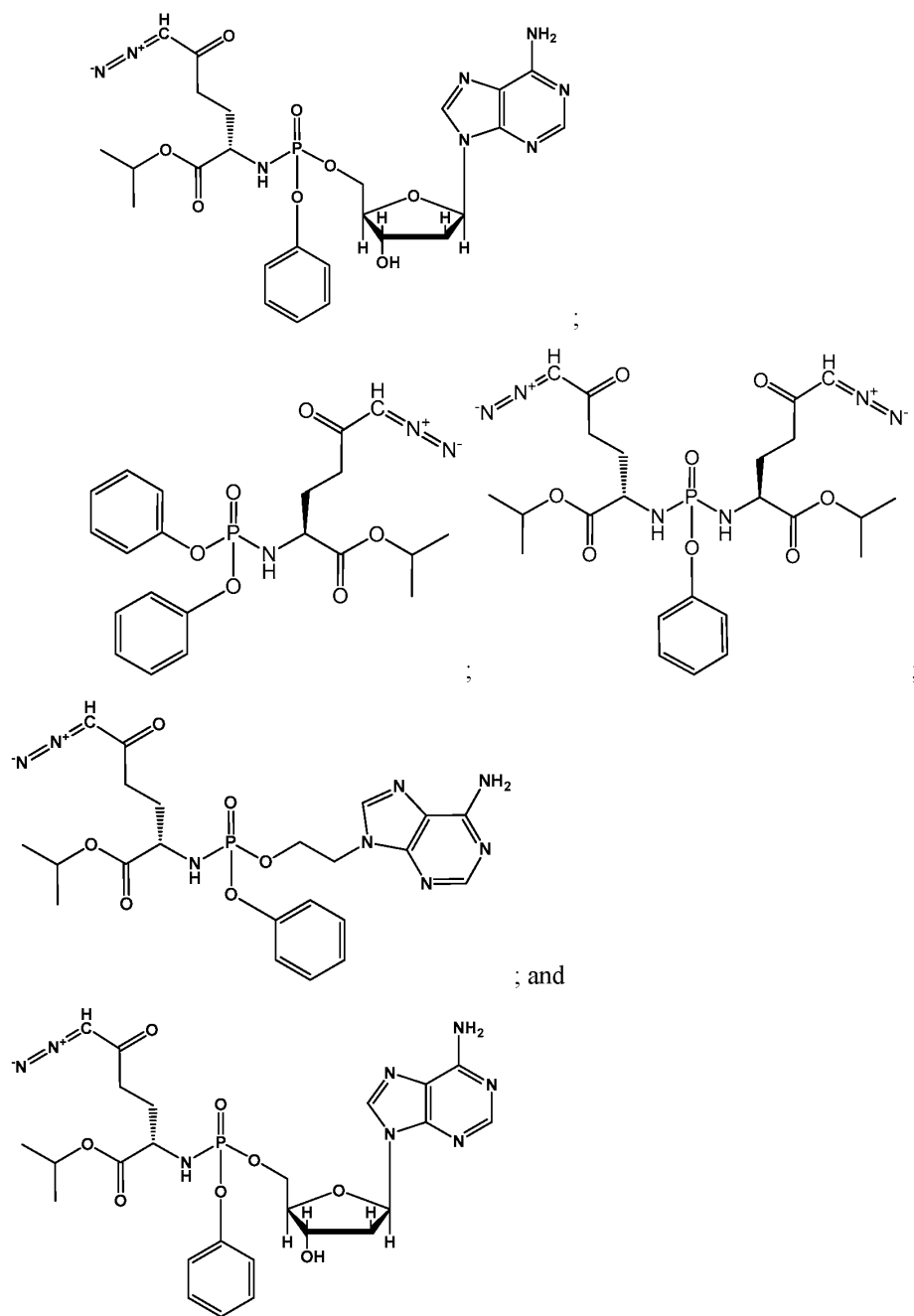
15



In such embodiments, it should be appreciated that the terminal carboxy
 5 groups of the compounds of formula (I) shown above can be used to form salts. In an
 exemplary embodiment, a salt of any of the compounds of formula (I) shown above
 can be formed when an H is replaced by NEt_3 , as will be appreciated by those skilled
 in the art.

In some embodiments, R_2 of formula (I) is $-\text{P}(=\text{O})(\text{OR}_7)_n(\text{NHR}_9)_o$, wherein: n
 10 is 2 and o is 0; n is 1 and o is 1; or n is 0 and o is 2.

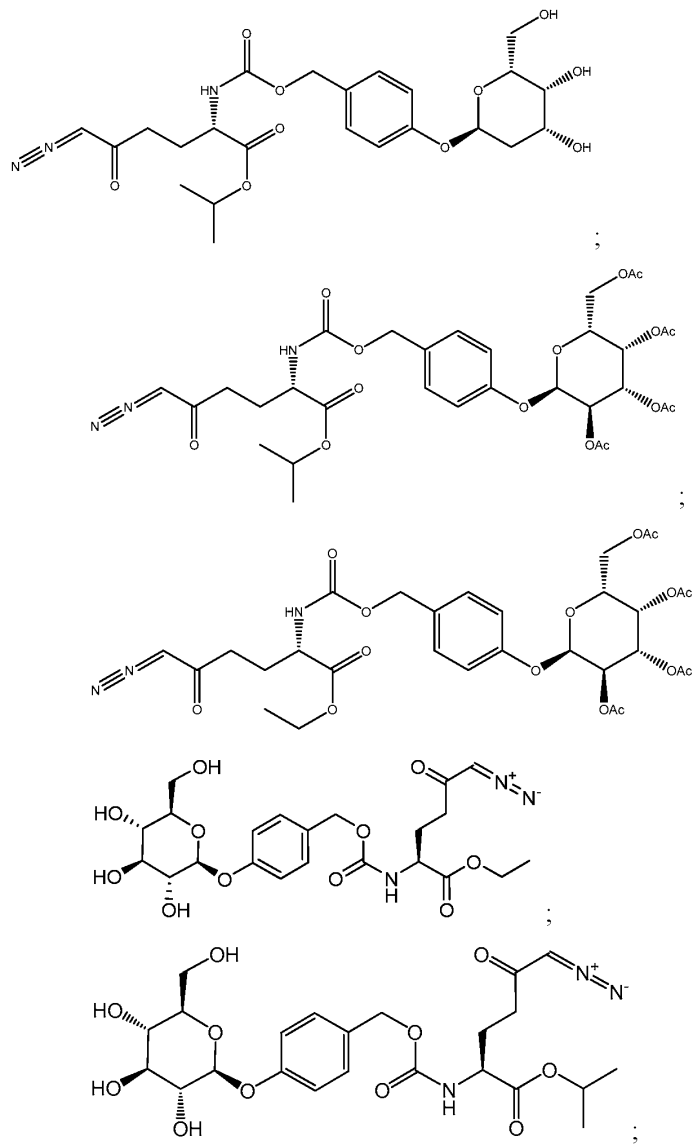
In particular embodiments, the prodrug compound of formula (I) is selected
 from the group consisting of:

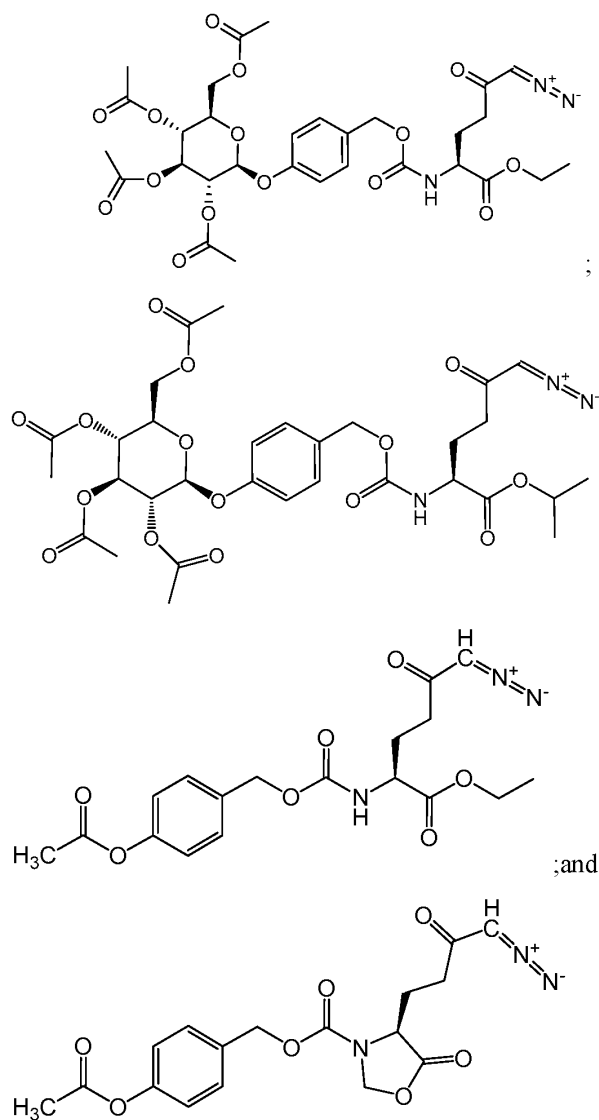


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In some embodiments, R₂ of formula (I) is -C(=O)-Y-(CR₃R₄)_m-Ar-O-C(=O)-R₈ or -C(=O)-Y-(CR₃R₄)_m-Ar-O-R₈; wherein: Y is -O-; m is 0; and Ar is benzyl.

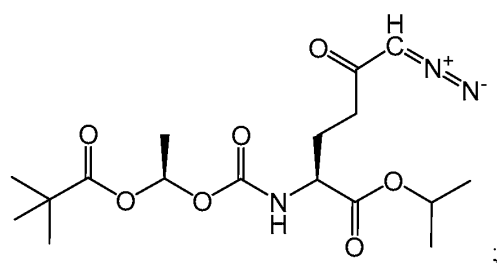
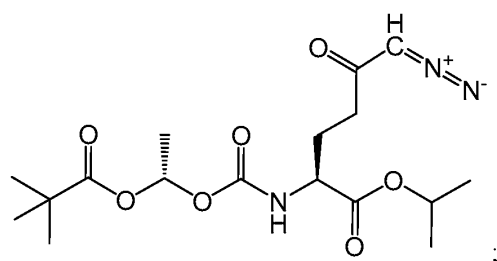
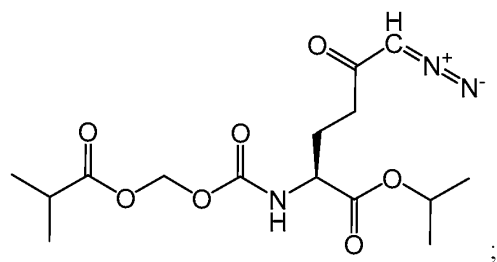
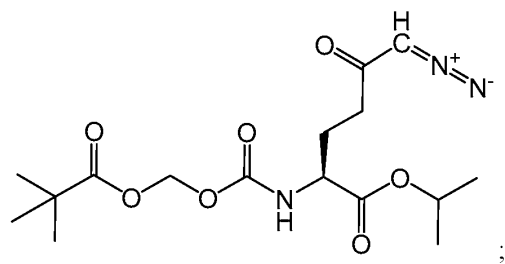
In particular embodiments, the prodrug compound of formula (I) is selected from the group consisting of:

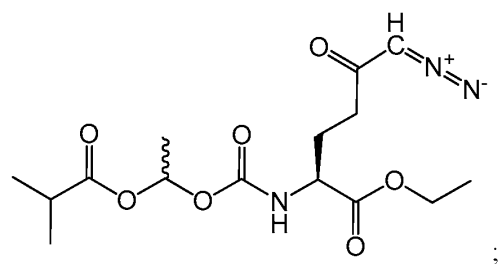
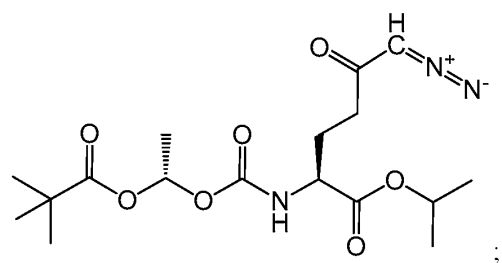
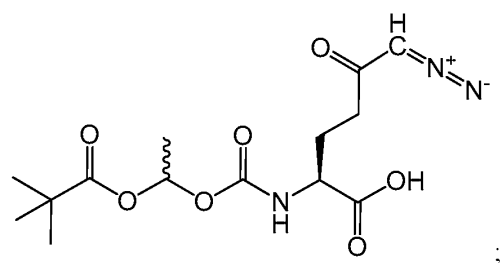
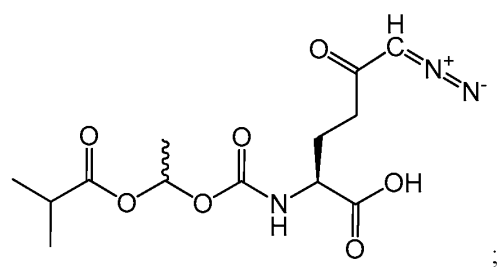
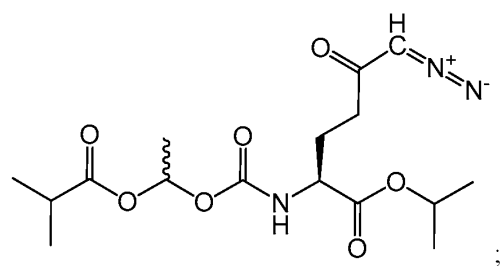




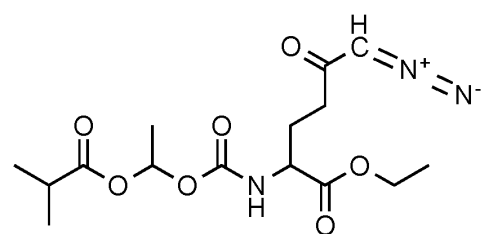
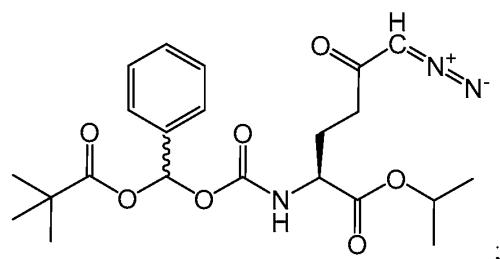
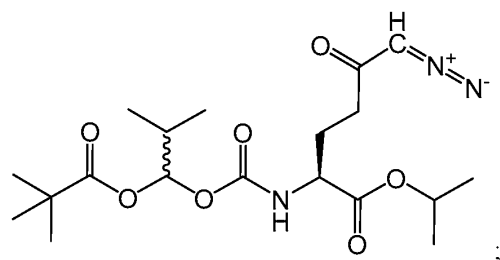
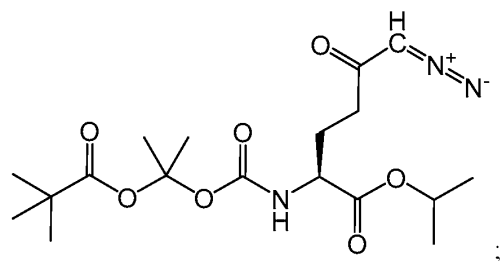
- 5 In some embodiments, R_2 of formula (I) is $-C(=O)-O-(CR_3R_4)_m-O-C(=O)-R_{10}$; wherein: (i) m is 1; R_3 is H; and R_4 is methyl, *i*Pr, or aryl; (ii) m is 1; R_3 and R_4 are each H; or (vi) m is 1; R_3 and R_4 are each methyl.

In particular embodiments, the prodrug compound of formula (I) is selected from the group consisting of:



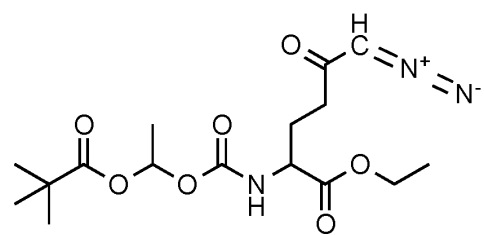


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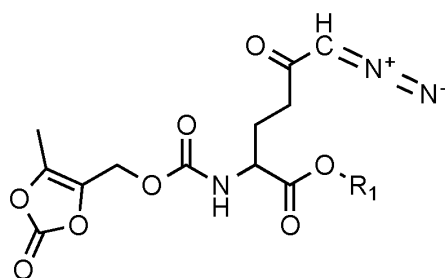


5

and

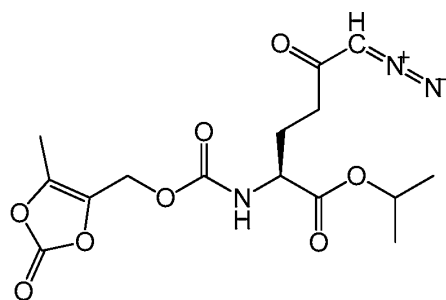
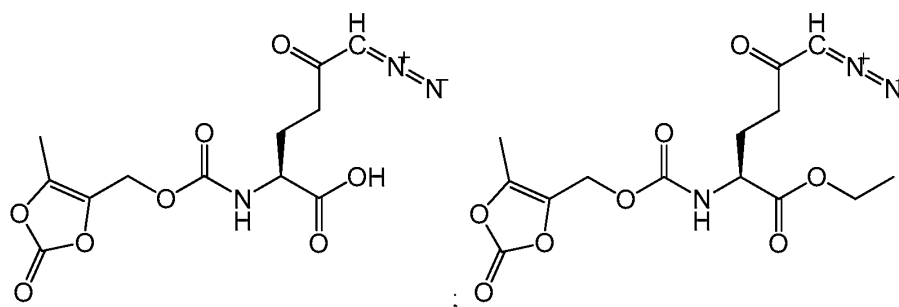


In particular embodiments, the prodrug compound of formula (I) is of formula:



, wherein R_1 is as defined above.

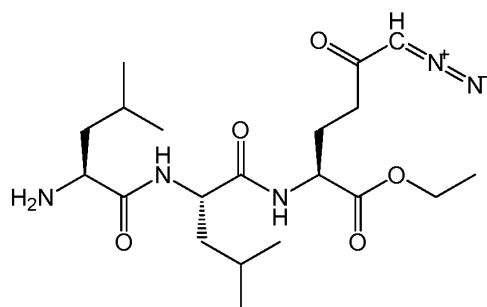
In particular embodiments, the prodrug compound of formula (I) is selected from the group consisting of:



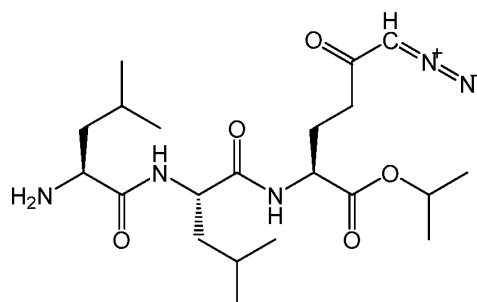
5 ; and

In some embodiments, R_2 of formula (I) is $-C(=O)-Y-(CR_3R_4)_m-NR_5R_6$; wherein Y is a bond; each m is 1; each R_5 is H; each R_6 is independently H or $-C(=O)-(CR_3R_4)_m-NR_5R_6$.

In particular embodiments, the prodrug compound of formula (I) is selected from the group consisting of:

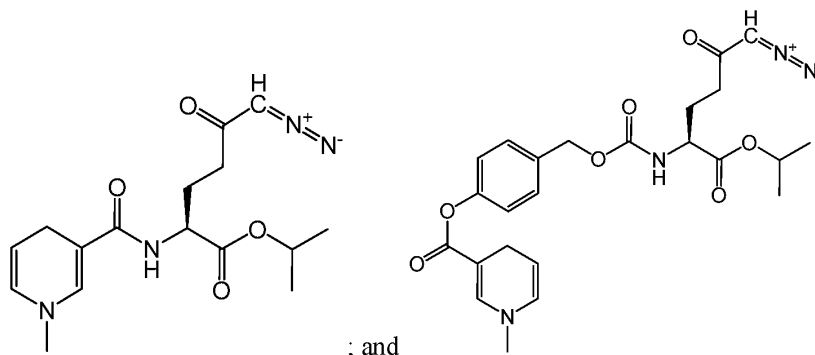


; and



In some embodiments, R_2 of formula (I) is $-C(=O)-Ar$,
or $-C(=O)-Y-(CR_3R_4)_m-Ar-O-C(=O)-Ar$.

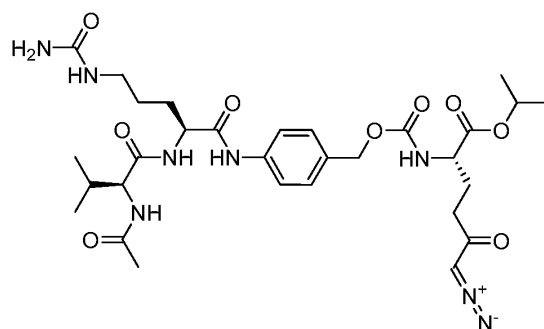
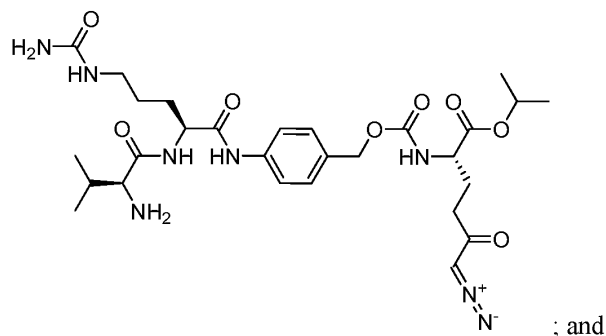
- 5 In particular embodiments, the prodrug compound of formula (I) is selected from the group consisting of:



; and

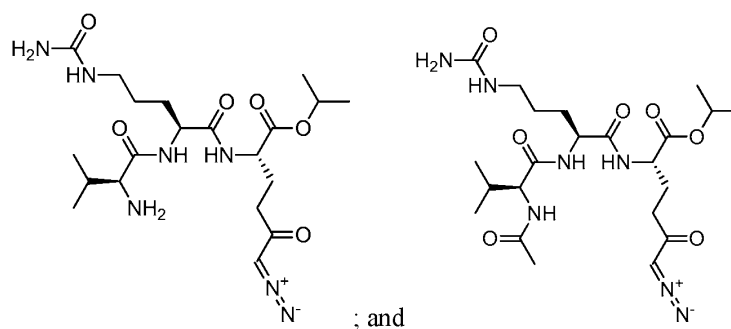
- In some embodiments, R_2 of formula (I) is $-C(=O)-Y-(CR_3R_4)_m-Ar-NR_5R_6$;
wherein: Y is O; each m is independently 0, 1, or 3; each R_3 is independently H, C_1-C_6
10 alkyl, or $-(CR_3R_4)_m-NR_5R_6$; each R_4 is H; each R_5 is independently H, $-C(=O)-$
 $(CR_3R_4)_m$, $-C(=O)-NR_5R_6$, or $-C(=O)-(CR_3R_4)_m-NR_5R_6$; each R_6 is H.

In particular embodiments, the prodrug compound of formula (I) is selected from the group consisting of:



- In some embodiments, R_2 of formula (I) is $-C(=O)-Y-(CR_3R_4)_m-NR_5R_6$, wherein: Y is a bond; m is 1, 2 or 3; each R_3 is independently H, C_1-C_6 alkyl, or $-(CR_3R_4)_m-NR_5R_6$; each R_4 is H; each R_5 is independently H, $-C(=O)-(CR_3R_4)_m$, $-C(=O)-(NR_5R_6)$, or $-C(=O)-(CR_3R_4)_m-NR_5R_6$; each R_6 is H.

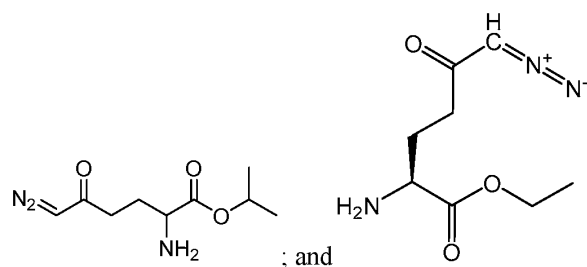
In particular embodiments, the prodrug compound of formula (I) is selected from the group consisting of:



10

In some embodiments, R_2 of formula (I) is H and R_1 is selected from the group consisting of alkyl and substituted alkyl.

In particular embodiments, the prodrug compound of formula (I) is selected from the group consisting of:



It should be appreciated that all of the compounds of formula (I) depicted in the structural formulas as prodrugs of DON are merely exemplary of prodrugs of glutamine analogs, such as L-DONV and aza-serine, that can be synthesized using the guidance of the presently disclosed subject matter.

II. Pharmaceutical Compositions and Administration

In another aspect, the present disclosure provides a pharmaceutical composition including one prodrug compound of formula (I), alone or in combination with one or more additional therapeutic agents in admixture with a pharmaceutically acceptable excipient. Accordingly, in some embodiments, the presently disclosed subject matter provides a pharmaceutical composition comprising a compound of formula (I), and a pharmaceutically acceptable carrier, diluent, or excipient. One of skill in the art will recognize that the pharmaceutical compositions include the pharmaceutically acceptable salts of the compounds described above.

Pharmaceutically acceptable salts are generally well known to those of ordinary skill in the art, and include salts of active compounds which are prepared with relatively nontoxic acids or bases, depending on the particular substituent moieties found on the compounds described herein. When compounds of the present disclosure contain relatively acidic functionalities, base addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired base, either neat or in a suitable inert solvent or by ion exchange, whereby one basic counterion (base) in an ionic complex is substituted for another. Examples of pharmaceutically acceptable base addition salts include sodium, potassium, calcium, ammonium, organic amino, or magnesium salt, or a similar salt.

When compounds of the present disclosure contain relatively basic functionalities, acid addition salts can be obtained by contacting the neutral form of

such compounds with a sufficient amount of the desired acid, either neat or in a suitable inert solvent or by ion exchange, whereby one acidic counterion (acid) in an ionic complex is substituted for another. Examples of pharmaceutically acceptable acid addition salts include those derived from inorganic acids like hydrochloric, hydrobromic, nitric, carbonic, monohydrogencarbonic, phosphoric, monohydrogenphosphoric, dihydrogenphosphoric, sulfuric, monohydrogensulfuric, hydriodic, or phosphorous acids and the like, as well as the salts derived from relatively nontoxic organic acids like acetic, propionic, isobutyric, maleic, malonic, benzoic, succinic, suberic, fumaric, lactic, mandelic, phthalic, benzenesulfonic, p-toluenesulfonic, citric, tartaric, methanesulfonic, and the like. Also included are salts of amino acids, such as arginate and the like, and salts of organic acids like glucuronic or galactunoric acids and the like (see, for example, Berge et al, "Pharmaceutical Salts," Journal of Pharmaceutical Science, 1977, 66, 1-19). Certain specific compounds of the present disclosure contain both basic and acidic functionalities that allow the compounds to be converted into either base or acid addition salts.

Accordingly, pharmaceutically acceptable salts suitable for use with the presently disclosed subject matter include, by way of example but not limitation, acetate, benzenesulfonate, benzoate, bicarbonate, bitartrate, bromide, calcium edetate, carnsylate, carbonate, citrate, edetate, edisylate, estolate, esylate, fumarate, gluceptate, gluconate, glutamate, glycollylarsanilate, hexylresorcinate, hydrabamine, hydrobromide, hydrochloride, hydroxynaphthoate, iodide, isethionate, lactate, lactobionate, malate, maleate, mandelate, mesylate, mucate, napsylate, nitrate, pamoate (embonate), pantothenate, phosphate/diphosphate, polygalacturonate, salicylate, stearate, subacetate, succinate, sulfate, tannate, tartrate, or teoclate. Other pharmaceutically acceptable salts may be found in, for example, Remington: The Science and Practice of Pharmacy (20th ed.) Lippincott, Williams & Wilkins (2000).

In particular embodiments, the salt is a tri(hydrocarbyl)ammonium or tetra(hydrocarbyl)ammonium salt. In yet more particular embodiments, the salt is selected from the group consisting of a tri(C₁-C₈-alkyl)ammonium, tetra(C₁-C₈-alkyl)ammonium, triphenylammonium, tri(hydroxy-C₁-C₈-alkyl)ammonium, and tetra(hydroxy-C₁-C₈-alkyl)ammonium salt. In even more particular embodiments, the salt is selected from the group consisting of a trimethylammonium, triethylammonium, tri(hydroxyethyl)ammonium, tripropylammonium, and tri(hydroxypropyl)ammonium salt.

In therapeutic and/or diagnostic applications, the compounds of the disclosure can be formulated for a variety of modes of administration, including oral (sublingual, buccal), peroral, sublingual, systemic and topical or localized administration.

Techniques and formulations generally may be found in Remington: The Science and Practice of Pharmacy (20th ed.) Lippincott, Williams & Wilkins (2000).

Depending on the specific conditions being treated, such agents may be formulated into liquid (e.g., solutions, suspensions, or emulsions) or solid dosage forms (capsules or tablets) and administered systemically or locally. The agents may be delivered, for example, in a timed-, controlled, or sustained-slow release form as is known to those skilled in the art. Techniques for formulation and administration may be found in Remington: The Science and Practice of Pharmacy (20th ed.) Lippincott, Williams & Wilkins (2000). Suitable routes may include oral, buccal, by inhalation spray, sublingual, rectal, transdermal, vaginal, transmucosal, nasal or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intra-articular, intra-sternal, intra-synovial, intra-hepatic, intralesional, intracranial, intraperitoneal, intranasal, or intraocular injections or other modes of delivery. In some embodiments, the pharmaceutical composition is administered orally. In some embodiments, the pharmaceutical composition is administered intravenously. In some embodiments, the pharmaceutical composition is administered intramuscularly. In some embodiments, the pharmaceutical composition is administered intrathecally. In some embodiments, the pharmaceutical composition is administered subcutaneously.

For injection, the agents of the disclosure may be formulated and diluted in aqueous solutions, such as in physiologically compatible buffers, such as Hank's solution, Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

Use of pharmaceutically acceptable inert carriers to formulate the compounds herein disclosed for the practice of the disclosure into dosages suitable for systemic administration is within the scope of the disclosure. With proper choice of carrier and suitable manufacturing practice, the compositions of the present disclosure, in particular, those formulated as solutions, may be administered parenterally, such as by intravenous injection. The compounds can be formulated readily using

pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the disclosure to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a subject (e.g., patient) to be treated.

5 For nasal or inhalation delivery, the agents of the disclosure also may be formulated by methods known to those of skill in the art, and may include, for example, but not limited to, examples of solubilizing, diluting, or dispersing substances, such as saline; preservatives, such as benzyl alcohol; absorption promoters; and fluorocarbons.

10 Pharmaceutical compositions suitable for use in the present disclosure include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. Generally, the compounds according to the disclosure are effective
15 over a wide dosage range. For example, in the treatment of adult humans, dosages from 0.01 to 1000 mg, from 0.5 to 100 mg, from 1 to 50 mg per day, and from 5 to 40 mg per day are examples of dosages that may be used. A non-limiting dosage is 10 to 30 mg per day. The exact dosage will depend upon the route of administration, the form in which the compound is administered, the subject to be treated, the body
20 weight of the subject to be treated, the bioavailability of the compound(s), the adsorption, distribution, metabolism, and excretion (ADME) toxicity of the compound(s), and the preference and experience of the attending physician.

In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and
25 auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions.

Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipients, optionally grinding a resulting mixture, and
30 processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers, such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl- cellulose, sodium

carboxymethyl-cellulose (CMC), and/or polyvinylpyrrolidone (PVP: povidone). If desired, disintegrating agents may be added, such as the cross-linked polyvinylpyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol (PEG), and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dye-stuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations that can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin, and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler, such as lactose, binders, such as starches, and/or lubricants such, as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols (PEGs). In addition, stabilizers may be added.

III. Methods for Treating a Disease or Disorder

The presently disclosed compounds are, which are orally bioavailable, less toxic prodrugs of glutamine analogs that are glutamine antagonists, allow a clinically acceptable dosing paradigm for diseases or conditions wherein excess and/or aberrant glutamine activity is implicated. As used herein, the term "glutamine antagonist" refers to a glutamine analog that interferes with a glutamine metabolic pathway, e.g., the inhibition or blocking of a metabolic pathway downstream of glutamine in which glutamine acts as a precursor of one or more non-glutamine compounds. Examples of such metabolic pathways are well known (see, e.g., Hensley et al., "Glutamine and cancer: cell biology, physiology, and clinical opportunities" *J Clin Invest.* 2013; 123(9):3678-3684; DeBerardinis et al., "Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer" *Oncogene.* 2009; 29(3):313-324; and Medina et al., "Relevance of glutamine metabolism to tumor cell growth" *Mol Cell Biochem.* 1992; 113(1):1-15). In some contexts, the term glutamine antagonist also includes glutamine analogs that inhibit glutamine uptake by cells, thereby reducing its biological activity. Diseases or conditions wherein excess and/or aberrant glutamine

activity is implicated include, but are not limited to, infection, cancer, autoimmune diseases, and neurodegenerative or neurological diseases and other central nervous system disorders.

5 In general, the presently disclosed methods result in a decrease in the severity of a disease or condition in a subject. The term "decrease" is meant to inhibit, suppress, attenuate, diminish, arrest, or stabilize a symptom of a disease or condition.

As used herein, the terms "treat," "treating," "treatment," and the like refer to reducing or ameliorating a disease or condition, and/or symptoms associated therewith. It will be appreciated that, although not precluded, treating a disease or
10 condition does not require that the disorder, condition or symptoms associated therewith be completely eliminated.

Accordingly, in some embodiments, the presently disclosed subject matter provide a method for treating a disease or a condition, the method comprising administering to a subject in need of treatment thereof, a compound of formula (I), or
15 a pharmaceutical composition of any thereof, in an amount effective for treating the disease or condition.

The presently disclosed subject matter contemplates using a prodrug of formula (I), or a pharmaceutical composition comprising the prodrug of formula (I), optionally together with at least one antimicrobial agent (e.g., antibiotic, antiviral, and
20 the like), to treat an infection.

As used herein, "infection" refers to the invasion of a host organism's bodily tissues by disease-causing organisms, their multiplication, and the reaction of host tissues to these organisms and the toxins they produce. Infectious disease, such as infection by any bacteria or virus, is contemplated for treatment using a compound of
25 formula (I), or a pharmaceutical composition of any thereof.

In some embodiments, the infection comprises a bacterial infection. Antibacterial effects of DON have been demonstrated in *E. coli* (see Coggin et al., "6-Diazo-5-Oxo-L-Norleucine Inhibition of Escherichia coli, " *Journal of Bacteriology*. 1965; 86). In some embodiments, the compound of formula (I), or
30 pharmaceutical composition of any thereof, inhibits bacterial growth and/or survival.

In some embodiments, the infection comprises a viral infection. The antiviral effects of glutamine analogs, such as DON, have been demonstrated (see, e.g., Cinatl et al., "Antiviral effects of 6-diazo-5-oxo-L-norleucine on replication of herpes simplex virus type 1" *Antiviral Research*. 1997; 33:165-175; Nishio et al., "Antiviral

effect of 6-diazo-5-oxo-L-norleucine, antagonist of γ -glutamyl transpeptidase, on replication of human parainfluenza virus type 2," *Journal of General Virology*. 1990; 71:61-67). Examples of viral infections contemplated for treatment using a compound of formula (I), or a pharmaceutical composition of any thereof include, without limitation, herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), human cytomegalovirus (HCMV), human parainfluenza virus type 2 (HPIV-2), Maloney leukemia virus (MLV), mumps, paramyxovirus, poliovirus, reovirus type 3, respiratory syncytial virus (RSV), Sendai virus, and vesicular stomatitis virus (VSV).

10 In some embodiments, the compound of formula (I), or pharmaceutical composition of any thereof, inhibits viral replication. In some embodiments, the compound of formula (I), or pharmaceutical composition of any thereof, inhibits replication of herpes simplex virus type 1 (HSV-1). In some embodiments, the compound of formula (I), or pharmaceutical composition of any thereof, inhibits
15 replication of herpes simplex virus type 2 (HSV-2). In some embodiments, the compound of formula (I), or pharmaceutical composition of any thereof, inhibits replication of human cytomegalovirus (HCMV). In some embodiments, the compound of formula (I), or pharmaceutical composition of any thereof, inhibits replication of human parainfluenza virus type 2 (HPIV-2). In some embodiments, the
20 compound of formula (I), or pharmaceutical composition of any thereof, inhibits replication of Maloney leukemia virus (MLV). In some embodiments, the compound of formula (I), or pharmaceutical composition of any thereof, inhibits replication of mumps. In some embodiments, the compound of formula (I), or pharmaceutical composition of any thereof, inhibits replication of paramyxovirus. In some
25 embodiments, the compound of formula (I), or pharmaceutical composition of any thereof, inhibits replication of poliovirus. In some embodiments, the compound of formula (I), or pharmaceutical composition of any thereof, inhibits replication of reovirus type 3. In some embodiments, the compound of formula (I), or pharmaceutical composition of any thereof, inhibits replication of respiratory
30 syncytial virus (RSV). In some embodiments, the compound of formula (I), or pharmaceutical composition of any thereof, inhibits replication of Sendai virus. In some embodiments, the compound of formula (I), or pharmaceutical composition of any thereof, inhibits replication of vesicular stomatitis virus (VSV).

 In some embodiments, the viral infection is influenza. As used herein,

"influenza" refers to influenza A, B, or C, parainfluenza viruses, and any other influenza-like virus (see, e.g., U.S. Publication No. 2006/0276438, incorporated by reference herein in its entirety, which discloses using DON, and azaserine for treatment of influenza).

5 In an aspect, the presently disclosed subject matter involves the use of a compound of formula (I), or a pharmaceutical composition thereof, optionally together with an antiviral agent, for the manufacture of a medicament for treating a viral infection and/or inhibiting replication.

As used herein, "antiviral agent" includes a compound that inhibits the
10 replication of viruses in cells, tissues, or organisms. Examples of antiviral agents contemplated for use in combination with a prodrug of formula (I), or a pharmaceutical composition comprising a prodrug of formula (I) include, but are not limited to, Acyclovir (2-amino-1,9-dihydro-9-[(2-hydroxyethoxy)methyl]-6H-purin-6-one), Valacyclovir (L-valine, 2-[(2-amino-1,6-dihydro-6-oxo-9H-purin-9-yl)methoxy]ethyl ester, Pencyclovir (9-[4-hydroxy-3-(hydroxymethylbutyl)]guanine),
15 Famcyclovir (2-[2-(amino-9H-purin-9-yl)]ethyl-1,3-propanediol diacetate), Ribavirin (1-beta-D-ribofuranosyl-1-H-1,2,4-triazol-3-carboxamide), Lamivudine ((2R,cis)-4-amino-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidine-2-one), Amantadine (1-amantanamine hydrochloride), and Rimantadine (α -methyltricyclo
20 (3.3.1.1/3.7 decane-1-methylamine hydrochloride).

The presently disclosed subject matter contemplates using a prodrug of formula (I), or a pharmaceutical composition comprising the prodrug of formula (I), optionally together with at least one chemotherapeutic agent, at least one radiotherapeutic agent, and/or at least one immunotherapeutic agent to treat cancer.
25 In some embodiments, such treatment includes treatment with any combination of radiotherapy, immunotherapy, photodynamic therapy, proton therapy, and/or surgery.

A "chemotherapeutic agent" is used to connote a compound or composition that is administered in the treatment of cancer. Chemotherapeutic agents contemplated for use in combination with a prodrug of formula (I), or a
30 pharmaceutical composition comprising a prodrug of formula (I) include, but are not limited to, alkylating agents, such as thiotepea and cyclophosphamide; alkyl sulfonates, such as busulfan, improsulfan and piposulfan; aziridines, such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramidate,

triethylenethiophosphoramidate and trimethylololomelamime; nitrogen mustards, such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas, such as

5 carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics, such as aclacinomysins, actinomycin, anthracycline, azaserine, bleomycins, cactinomycin, calicheamicin, carabycin, caminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid,

10 nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites, such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues, such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs, such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs,

15 such as ancitabine, azacitidine, 6-azauridine, carmofur, cytosine arabinoside, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens, such as calusterone, dromostanolone propionate, epitestosterone, mepitostane, testolactone; anti-adrenals, such as aminoglutethimide, mitotane, trilostane; folic acid replenishers, such as folinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid;

20 amsacrine; bestabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguanine; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK; razoxane; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2''-

25 trichlorotriethylamine; urethane; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside (Ara-C); taxoids, e.g., paclitaxel and docetaxel; chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; platinum analogs, such as cisplatin and carboplatin; vinblastine; platinum; etoposide; ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine;

30 novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT11; topoisomerase inhibitor RFS 2000; difluoromethylornithine; retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Chemotherapeutic agents also include anti-hormonal agents that act to regulate or inhibit hormone action on tumors, such as anti-estrogens

including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and anti-androgens, such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives
5 of any of the above.

In some embodiments, the chemotherapeutic agent is a topoisomerase inhibitor. Topoisomerase inhibitors are chemotherapy agents that interfere with the action of a topoisomerase enzyme (e.g., topoisomerase I or II). Topoisomerase inhibitors include, but are not limited to, doxorubicin HCl, daunorubicin citrate,
10 mitoxantrone HCl, actinomycin D, etoposide, topotecan HCl, teniposide, and irinotecan, as well as pharmaceutically acceptable salts, acids, or derivatives of any of these.

In some embodiments, the chemotherapeutic agent is an anti-metabolite. An anti-metabolite is a chemical with a structure that is similar to a metabolite required
15 for normal biochemical reactions, yet different enough to interfere with one or more normal functions of cells, such as cell division. Anti-metabolites include, but are not limited to, gemcitabine, fluorouracil, capecitabine, methotrexate sodium, raltitrexed, pemetrexed, tegafur, cytosine arabinoside, thioguanine, 5-azacytidine, 6-mercaptopurine, azathioprine, 6-thioguanine, pentostatin, fludarabine phosphate, and
20 cladribine, as well as pharmaceutically acceptable salts, acids, or derivatives of any of these.

In certain embodiments, the chemotherapeutic agent is an antimetabolic agent, including, but not limited to, agents that bind tubulin. In some embodiments, the agent is a taxane. In certain embodiments, the agent is paclitaxel or docetaxel, or a
25 pharmaceutically acceptable salt, acid, or derivative of paclitaxel or docetaxel. In certain alternative embodiments, the antimetabolic agent comprises a vinca alkaloid, such as vincristine, vinblastine, vinorelbine, or vindesine, or pharmaceutically acceptable salts, acids, or derivatives thereof.

As used herein, the term “immunotherapeutic agent” refers to a molecule that
30 can aid in the treatment of a disease by inducing, enhancing, or suppressing an immune response in a cell, tissue, organ or subject. Examples of immunotherapeutic agents contemplated for use in combination with a prodrug of formula (I), or a pharmaceutical composition comprising a prodrug of formula (I) include, but are not limited to, immune checkpoint molecules (e.g., antibodies to immune checkpoint

proteins), interleukins (e.g., IL-2, IL-7, IL-12, IL-15), cytokines (e.g., interferons, G-CSF, imiquimod), chemokines (e.g., CCL3, CCL26, CXCL7), vaccines (e.g., peptide vaccines, dendritic cell (DC) vaccines, EGFRvIII vaccines, mesothelin vaccine, G-VAX, listeria vaccines), and adoptive T cell therapy including chimeric antigen
5 receptor T cells (CAR T cells).

As used herein, "radiotherapeutic agent" means an agent which may be used in radiotherapy that acts through damaging cells (e.g., malignant cells) as a target through radiation irradiation. An exemplary radiotherapeutic agent contemplated for use in combination with a prodrug of formula (I), or a pharmaceutical composition
10 comprising a prodrug of formula (I) is the titanium peroxide contained in the substrate particle which generates a hydroxyl radical through radiation irradiation, and the hydroxyl radical exerts an action of attacking a target, as described in U.S. Publication No. 2013/0017266, which is incorporated by reference herein in its entirety.

As used herein, a "cancer" in a patient refers to the presence of cells
15 possessing characteristics typical of cancer-causing cells, for example, uncontrolled proliferation, loss of specialized functions, immortality, significant metastatic potential, significant increase in anti-apoptotic activity, rapid growth and proliferation rate, and certain characteristic morphology and cellular markers. In some circumstances, cancer cells will be in the form of a tumor; such cells may exist locally
20 within an animal, or circulate in the blood stream as independent cells, for example, leukemic cells. A "tumor," as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all precancerous and cancerous cells and tissues. A "solid tumor," as used herein, is an abnormal mass of tissue that generally does not contain cysts or liquid areas. A solid tumor may be in the brain,
25 colon, breasts, prostate, liver, kidneys, lungs, esophagus, head and neck, ovaries, cervix, stomach, colon, rectum, bladder, uterus, testes, and pancreas, as non-limiting examples. In some embodiments, the solid tumor regresses or its growth is slowed or arrested after the solid tumor is treated with the presently disclosed methods. In other embodiments, the solid tumor is malignant. In some embodiments, the cancer
30 comprises Stage 0 cancer. In some embodiments, the cancer comprises Stage I cancer. In some embodiments, the cancer comprises Stage II cancer. In some embodiments, the cancer comprises Stage III cancer. In some embodiments, the cancer comprises Stage IV cancer. In some embodiments, the cancer is refractory and/or metastatic. For example, the cancer may be refractory to treatment with

- radiotherapy, chemotherapy or monotreatment with immunotherapy. Cancer as used herein includes newly diagnosed or recurrent cancers, including without limitation, acute lymphoblastic leukemia, acute myelogenous leukemia, advanced soft tissue sarcoma, brain cancer, metastatic or aggressive breast cancer, breast carcinoma, bronchogenic carcinoma, choriocarcinoma, chronic myelocytic leukemia, colon carcinoma, colorectal carcinoma, Ewing's sarcoma, gastrointestinal tract carcinoma, glioma, glioblastoma multiforme, head and neck squamous cell carcinoma, hepatocellular carcinoma, Hodgkin's disease, intracranial ependymoblastoma, large bowel cancer, leukemia, liver cancer, lung carcinoma, Lewis lung carcinoma, lymphoma, malignant fibrous histiocytoma, a mammary tumor, melanoma, mesothelioma, neuroblastoma, osteosarcoma, ovarian cancer, pancreatic cancer, a pontine tumor, premenopausal breast cancer, prostate cancer, rhabdomyosarcoma, reticulum cell sarcoma, sarcoma, small cell lung cancer, a solid tumor, stomach cancer, testicular cancer, and uterine carcinoma.
- 15 In some embodiments, the cancer is acute leukemia. In some embodiments, the cancer is acute lymphoblastic leukemia. In some embodiments, the cancer is acute myelogenous leukemia. In some embodiments, the cancer is advanced soft tissue sarcoma. In some embodiments, the cancer is a brain cancer. In some embodiments, the cancer is breast cancer (e.g., metastatic or aggressive breast cancer).
- 20 In some embodiments, the cancer is breast carcinoma. In some embodiments, the cancer is bronchogenic carcinoma. In some embodiments, the cancer is choriocarcinoma. In some embodiments, the cancer is chronic myelocytic leukemia. In some embodiments, the cancer is a colon carcinoma (e.g., adenocarcinoma). In some embodiments, the cancer is colorectal cancer (e.g., colorectal carcinoma). In
- 25 some embodiments, the cancer is Ewing's sarcoma. In some embodiments, the cancer is gastrointestinal tract carcinoma. In some embodiments, the cancer is a glioma. In some embodiments, the cancer is glioblastoma multiforme. In some embodiments, the cancer is head and neck squamous cell carcinoma. In some embodiments, the cancer is hepatocellular carcinoma. In some embodiments, the cancer is Hodgkin's disease.
- 30 In some embodiments, the cancer is intracranial ependymoblastoma. In some embodiments, the cancer is large bowel cancer. In some embodiments, the cancer is leukemia. In some embodiments, the cancer is liver cancer. In some embodiments, the cancer is lung cancer (e.g., lung carcinoma). In some embodiments, the cancer is Lewis lung carcinoma. In some embodiments, the cancer is lymphoma. In some

embodiments, the cancer is malignant fibrous histiocytoma. In some embodiments, the cancer comprises a mammary tumor. In some embodiments, the cancer is melanoma. In some embodiments, the cancer is mesothelioma. In some embodiments, the cancer is neuroblastoma. In some embodiments, the cancer is osteosarcoma. In some embodiments, the cancer is ovarian cancer. In some embodiments, the cancer is pancreatic cancer. In some embodiments, the cancer comprises a pontine tumor. In some embodiments, the cancer is premenopausal breast cancer. In some embodiments, the cancer is prostate cancer. In some embodiments, the cancer is rhabdomyosarcoma. In some embodiments, the cancer is reticulum cell sarcoma. In some embodiments, the cancer is sarcoma. In some embodiments, the cancer is small cell lung cancer. In some embodiments, the cancer comprises a solid tumor. In some embodiments, the cancer is stomach cancer. In some embodiments, the cancer is testicular cancer. In some embodiments, the cancer is uterine carcinoma.

15 In some embodiments, the cancer comprises a glutamine-dependent cancer in which glutamine is an important fuel source for cellular energy in the cancer (e.g., hematopoietic tumors, hepatomas, Ehrlich carcinoma (see Huber et al., "Uptake of glutamine antimetabolites 6-diazo-5-oxo-L-norleucine (DON) in sensitive and resistant tumor cell lines," *Int. J. Cancer*. 1988; 41:752-755)).

20 In some embodiments, the cancer is a Myc-dependent cancer. As used herein, "Myc-dependent cancer" refers to a cancer exhibiting activation, overexpression and/or amplification of c-Myc. In some contexts, a "Myc-dependent cancer" is a cancer in which c-Myc plays a role in increased glutamine metabolism in the cancer cells. Examples of Myc-dependent cancers include, without limitation, lymphoma, neuroblastoma, and small cell lung cancer.

25 In some embodiments, the cancer is an mTORC1-dependent cancer. As used herein, "mTORC1-dependent cancer" refers to a cancer in which mTORC1 is activated in a glutamine-dependent manner, i.e., that is mTORC1 plays a role in increased glutamine metabolism in the cancer cells.

30 The presently disclosed subject matter contemplates using a prodrug of formula (I), or a pharmaceutical composition comprising the prodrug of formula (I), optionally together with at least one immunosuppressant and/or anti-inflammatory agent, to treat an autoimmune disease, immune disorder, or inflammatory disorder.

As used herein, "immunosuppressant agent" means an agent which may be

used in immunotherapy to reduce or prevent an immune response in a cell, organ, tissue, or subject. Examples of immunosuppressant agents contemplated for use in combination with a prodrug of formula (I), or a pharmaceutical composition comprising a prodrug of formula (I) include corticosteroids, calcineurin inhibitors, antiproliferative agents, SIP receptor agonists, kinase inhibitors, monoclonal antilymphocyte antibodies and polyclonal antilymphocyte antibodies. Non-limiting examples of corticosteroids include Prednisone (Deltasone® and Orasone®) and Methylprednisolone (SoluMedrol®). Non-limiting examples of calcineurin inhibitors include Cyclosporine (Cyclosporin A, SangCya, Sandimmune®, Neoral®, Gengraf®), ISA, Tx247, ABT-281, ASM 981 and Tacrolimus (Prograf®, FK506). Non-limiting examples of antiproliferative agents include Mycophenolate Mofetil (CellCept®), Azathioprene (Imuran®), and Sirolimus (Rapamune®). Non-limiting examples of SIP receptor agonists include FTY 720 or analogues thereof. Non-limiting examples of kinase inhibitors include mTOR kinase inhibitors, which are compounds, proteins or antibodies that target, decrease or inhibit the activity and/or function of members of the serine/threonine mTOR family. These include, without limitation, CCI-779, ABT578, SAR543, rapamycin and derivatives or analogs thereof, including 40-O-(2-hydroxyethyl)-rapamycin, rapalogs, including AP23573, AP23464, AP23675 and AP23841 from Ariad, Everolimus (CERTICAN, RAD001), biolimus 7, biolimus 9 and sirolimus (RAPAMUNE). Kinase inhibitors also include protein kinase C inhibitors, which include the compounds described the PCT publications WO 2005/097108 and WO 2005/068455, which are herein incorporated by reference in their entireties. Non-limiting examples of monoclonal antilymphocyte antibodies include Muromonab-CD3 (Orthoclone OKT3®), Interleukin-2 Receptor Antagonist (Basiliximab, Simulect®), and Daclizumab (Zenapax®). Non-limiting examples of polyclonal antilymphocyte antibodies include Antithymocyte globulin-equine (Atgam®) and Antithymocyte globulin-rabbit (RATG, Thymoglobulin®). Other immunosuppressants include, without limitation, SERP-1, a serine protease inhibitor produced by malignant rabbit fibroma virus (MRV) and myxoma virus (MYX), described in US Patent Publication No. 2004/0029801, which is incorporated herein by reference.

As used herein, "anti-inflammatory agent" refers to an agent that may be used to prevent or reduce an inflammatory response or inflammation in a cell, tissue, organ, or subject. Exemplary anti-inflammatory agents contemplated for use in combination

- with a prodrug of formula (I), or a pharmaceutical composition comprising a prodrug of formula (I) include, without limitation, steroidal anti-inflammatory agents, a nonsteroidal anti-inflammatory agent, or a combination thereof. In some embodiments, anti-inflammatory agents include clobetasol, alclofenac, alclometasone
- 5 dipropionate, algestone acetone, alpha amylase, amcinafal, amcinafide, amfenac sodium, amiprilose hydrochloride, anakinra, aniolac, anitrazafen, apazone, balsalazide disodium, bendazac, benoxaprofen, benzydamine hydrochloride, bromelains, broperamole, budesonide, carprofen, cicloprofen, cintazone, cliprofen, clobetasol propionate, clobetasone butyrate, clopirac, cloticasone propionate,
 - 10 cormethasone acetate, cortodoxone, deflazacort, desonide, desoximetasone, dexamethasone, dexamethasone acetate, dexamethasone dipropionate, diclofenac potassium, diclofenac sodium, diflorasone diacetate, diflumidone sodium, diflunisal, difluprednate, diftalone, dimethyl sulfoxide, drocinonide, endrysone, enlimomab, enolicam sodium, eprizole, etodolac, etofenamate, felbinac, fenamole, fenbufen,
 - 15 fenclofenac, fenclorac, fendosal, fempipalone, fentiazac, flazalone, fluazacort, flufenamic acid, flumizole, flunisolid acetate, flunixin, flunixin meglumine, fluocortin butyl, fluorometholone acetate, fluquazone, flurbiprofen, fluretofen, fluticasone propionate, furaprofen, furobufen, halcinonide, halobetasol propionate, halopredone acetate, ibufenac, ibuprofen, ibuprofen aluminum, ibuprofen piconol,
 - 20 ilonidap, indomethacin, indomethacin sodium, indoprofen, indoxole, intrazole, isoflupredone acetate, isoxepac, isoxicam, ketoprofen, lofemizole hydrochloride, lomoxicam, loteprednol etabonate, meclofenamate sodium, meclofenamic acid, meclorisone dibutyrate, mefenamic acid, mesalamine, meseclazone, methylprednisolone suleptanate, momiflumate, nabumetone, naproxen,
 - 25 sodium, naproxol, nimazone, olsalazine sodium, orgotein, orpanoxin, oxaprozin, oxyphenbutazone, paranyline hydrochloride, pentosan polysulfate sodium, phenbutazone sodium glycerate, pirfenidone, piroxicam, piroxicam cinnamate, piroxicam olamine, pirprofen, prednazate, prifelone, prodolic acid, proquazone, proxazole, proxazole citrate, rimexolone, romazarit, salcolex, salnacedin, salsalate,
 - 30 sanguinarium chloride, seclazone, sermetacin, sudoxicam, sulindac, suprofen, talmetacin, talniflumate, talosalate, tebufelone, tenidap, tenidap sodium, tenoxicam, tesicam, tesimide, tetrydamine, tiopinac, tixocortol pivalate, tolmetin, tolmetin sodium, triclone, triflumidate, zidometacin, zomepirac sodium, aspirin (acetylsalicylic acid), salicylic acid, corticosteroids, glucocorticoids, tacrolimus,

pimecorlimus, prodrugs thereof, co-drugs thereof, and combinations thereof. The anti-inflammatory agent may also be a biological inhibitor of proinflammatory signaling molecules including antibodies to such biological inflammatory signaling molecules.

5 Autoimmunity is the failure of an organism to recognize its own constituent parts (down to the sub-molecular levels) as "self," which results in an immune response against its own cells and tissues. Any disease that results from such an aberrant immune response is termed an autoimmune disease. An unwanted immune response may be, for example, immune responses associated with an autoimmune disorder, allergies, or inflammatory disorders. The term "immune disorders" are
10 diseases involving the immune system that can include but not be limited to allergies, autoimmune diseases, immune complex diseases, immunodeficiency diseases and cancers of the immune system. In some embodiments, the autoimmune disease, immune disorder, or inflammatory disorder is multiple sclerosis.

15 The presently disclosed subject matter contemplates using a prodrug of formula (I), or a pharmaceutical composition comprising the prodrug of formula (I), optionally together with at least one neuroprotective agent and/or at least one neurotrophic factor, and/or at least one neuroregenerative agent, to treat a neurodegenerative or neurological disorder or disease.

20 A "neurodegenerative disorder" is a disease, disorder, or condition that is characterized by the progressive loss of the structure or function of neurons (e.g., degeneration or dysfunction of neurons or other neural cells). Glutaminase-catalyzed hydrolysis of glutamine to glutamate is a predominant source of brain glutamate. Normal central nervous system (CNS) synaptic transmission uses glutamate as the
25 major excitatory amino acid neurotransmitter. Excessive glutamatergic signaling, known as excitotoxicity, is believed to cause CNS damage in various neurodegenerative diseases, such as stroke, amyotrophic lateral sclerosis (ALS), Huntington's disease, Alzheimer's disease, and HIV-associated dementia. Accordingly, without wishing to be bound by theory, it is believed that the presently
30 disclosed prodrugs can be used to treat such neurodegenerative diseases, for example, by inhibiting glutaminase to ameliorate the CNS damage resulting from excitotoxicity due to excessive glutamatergic signaling.

 In particular embodiments, the neurodegenerative disorder is multiple sclerosis (MS). DON has been shown to be effective in ameliorating experimental

autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS) (see, e.g., Shijie, et al., "Blockade of glutamate release from microglia attenuates experimental autoimmune encephalomyelitis in mice," *Tohoku J. Exp. Med.* 2009; 217:87-92). In particular embodiments, the neurodegenerative disorder is HIV-associated dementia (HAD). In particular embodiments, the neurodegenerative disorder is ischemia (e.g., transient ischemic brain injury). In particular embodiments, the neurodegenerative disorder is stroke. In particular embodiments, the neurodegenerative disorder is amyotrophic lateral sclerosis (ALS). In particular embodiments, the neurodegenerative disorder is Huntington's disease. In particular
10 embodiments, the neurodegenerative disorder is Alzheimer's disease.

In some embodiments, the presently disclosed subject matter provides methods for inhibiting the excess and/or aberrant glutamine activity found in a subject with a disease or condition. As used herein, the term "inhibit" means to decrease or diminish the excess and/or aberrant glutamine activity found in a subject. The term
15 "inhibit" also may mean to decrease, suppress, attenuate, diminish, arrest, or stabilize the development or progression of a disease or condition. Inhibition may occur, for e.g., by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98%, 99%, or even 100% compared to an untreated control subject or a subject without the disease or disorder. As used herein, the term "excess glutamine activity" means an
20 increase in glutamine activity in a subject with a disease or condition as compared to the glutamine activity in a subject without a similar disease or condition, such as an increase of approximately 100%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000%, or more. As used herein, the term "aberrant glutamine activity" means a change in the biological activity of glutamine in a subject with a disease or
25 condition as compared to the glutamine activity in a subject without a similar disease or condition, such utilization of glutamine in the growth and/or proliferation of malignant, neoplastic, or other pathologic cellular processes.

In some embodiments, the disease or condition involves excess and/or aberrant glutamine activity. In such aspects, the method further comprises inhibiting
30 the excess and/or aberrant glutamine activity when the compound of formula (I), or the pharmaceutical composition of any thereof, is administered.

In another aspect, the presently disclosed subject matter involves the use of a compound of formula (I), or a pharmaceutical composition of any thereof, for treating a disease or condition. In some embodiments, the compound of formula (I), or the

pharmaceutical composition of any thereof is used to treat a disease or condition selected from the group consisting of an infection, cancer, an autoimmune disease, an inflammatory disease, and a neurodegenerative or neurological disease. In some embodiments, the compound of formula (I), or the pharmaceutical composition of any thereof is used to treat a disease or condition selected from the group consisting of multiple sclerosis, convulsions, epilepsy, and viral encephalitis. In some embodiments, the compound of formula (I), or the pharmaceutical composition of any thereof is used to treat a disease or condition that involves excess and/or aberrant glutamine activity. In such aspects, the use involves inhibiting the excess and/or aberrant glutamine activity when the compound of formula (I), or the pharmaceutical composition of any thereof, is used to treat the disease or condition.

IV. General Definitions

Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this presently described subject matter belongs.

While the following terms in relation to compounds of formula (I) are believed to be well understood by one of ordinary skill in the art, the following definitions are set forth to facilitate explanation of the presently disclosed subject matter. These definitions are intended to supplement and illustrate, not preclude, the definitions that would be apparent to one of ordinary skill in the art upon review of the present disclosure.

The terms substituted, whether preceded by the term “optionally” or not, and substituent, as used herein, refer to the ability, as appreciated by one skilled in this art, to change one functional group for another functional group on a molecule, provided that the valency of all atoms is maintained. When more than one position in any given structure may be substituted with more than one substituent selected from a specified group, the substituent may be either the same or different at every position. The substituents also may be further substituted (e.g., an aryl group substituent may have another substituent off it, such as another aryl group, which is further substituted at one or more positions).

Where substituent groups or linking groups are specified by their conventional

chemical formulae, written from left to right, they equally encompass the chemically identical substituents that would result from writing the structure from right to left, e.g., $-\text{CH}_2\text{O}-$ is equivalent to $-\text{OCH}_2-$; $-\text{C}(=\text{O})\text{O}-$ is equivalent to $-\text{OC}(=\text{O})-$; $-\text{OC}(=\text{O})\text{NR}-$ is equivalent to $-\text{NRC}(=\text{O})\text{O}-$, and the like.

5 When the term “independently selected” is used, the substituents being referred to (e.g., R groups, such as groups R_1 , R_2 , and the like, or variables, such as “m” and “n”), can be identical or different. For example, both R_1 and R_2 can be substituted alkyls, or R_1 can be hydrogen and R_2 can be a substituted alkyl, and the like.

10 The terms “a,” “an,” or “a(n),” when used in reference to a group of substituents herein, mean at least one. For example, where a compound is substituted with “an” alkyl or aryl, the compound is optionally substituted with at least one alkyl and/or at least one aryl. Moreover, where a moiety is substituted with an R substituent, the group may be referred to as “R-substituted.” Where a moiety is R-
15 substituted, the moiety is substituted with at least one R substituent and each R substituent is optionally different.

 A named “R” or group will generally have the structure that is recognized in the art as corresponding to a group having that name, unless specified otherwise herein. For the purposes of illustration, certain representative “R” groups as set forth
20 above are defined below.

 Descriptions of compounds of the present disclosure are limited by principles of chemical bonding known to those skilled in the art. Accordingly, where a group may be substituted by one or more of a number of substituents, such substitutions are selected so as to comply with principles of chemical bonding and to give compounds
25 which are not inherently unstable and/or would be known to one of ordinary skill in the art as likely to be unstable under ambient conditions, such as aqueous, neutral, and several known physiological conditions. For example, a heterocycloalkyl or heteroaryl is attached to the remainder of the molecule via a ring heteroatom in compliance with principles of chemical bonding known to those skilled in the art
30 thereby avoiding inherently unstable compounds.

 Unless otherwise explicitly defined, a “substituent group,” as used herein, includes a functional group selected from one or more of the following moieties, which are defined herein:

The term hydrocarbon, as used herein, refers to any chemical group comprising hydrogen and carbon. The hydrocarbon may be substituted or unsubstituted. As would be known to one skilled in this art, all valencies must be satisfied in making any substitutions. The hydrocarbon may be unsaturated, saturated, 5 branched, unbranched, cyclic, polycyclic, or heterocyclic. Illustrative hydrocarbons are further defined herein below and include, for example, methyl, ethyl, *n*-propyl, isopropyl, cyclopropyl, allyl, vinyl, *n*-butyl, *tert*-butyl, ethynyl, cyclohexyl, and the like.

Further, more generally, a "carbyl" refers to a carbon atom or a moiety 10 comprising one or more carbon atoms acting as a bivalent radical.

The term "alkyl," by itself or as part of another substituent, means, unless otherwise stated, a straight (i.e., unbranched) or branched chain, acyclic or cyclic hydrocarbon group, or combination thereof, which may be fully saturated, mono- or polyunsaturated and can include di- and multivalent groups, having the number of 15 carbon atoms designated (i.e., C₁-C₁₀ means one to ten carbons, including 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 carbons). In particular embodiments, the term "alkyl" refers to C₁₋₂₀ inclusive, including 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, and 20 carbons, linear (i.e., "straight-chain"), branched, or cyclic, saturated or at least partially and in some cases fully unsaturated (i.e., alkenyl and alkynyl) hydrocarbon 20 radicals derived from a hydrocarbon moiety containing between one and twenty carbon atoms by removal of a single hydrogen atom.

Representative saturated hydrocarbon groups include, but are not limited to, methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, isobutyl, *sec*-butyl, *tert*-butyl, *n*-pentyl, *sec*-pentyl, isopentyl, neopentyl, *n*-hexyl, *sec*-hexyl, *n*-heptyl, *n*-octyl, *n*-decyl, *n*- 25 undecyl, dodecyl, cyclohexyl, (cyclohexyl)methyl, cyclopropylmethyl, and homologs and isomers thereof.

"Branched" refers to an alkyl group in which a lower alkyl group, such as methyl, ethyl or propyl, is attached to a linear alkyl chain. "Lower alkyl" refers to an alkyl group having 1 to about 8 carbon atoms (i.e., a C₁₋₈ alkyl), e.g., 1, 2, 3, 4, 5, 6, 7, 30 or 8 carbon atoms. "Higher alkyl" refers to an alkyl group having about 10 to about 20 carbon atoms, e.g., 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 carbon atoms. In certain embodiments, "alkyl" refers, in particular, to C₁₋₈ straight-chain alkyls. In other embodiments, "alkyl" refers, in particular, to C₁₋₈ branched-chain alkyls.

Alkyl groups can optionally be substituted (a “substituted alkyl”) with one or more alkyl group substituents, which can be the same or different. The term “alkyl group substituent” includes but is not limited to alkyl, substituted alkyl, halo, arylamino, acyl, hydroxyl, aryloxy, alkoxy, alkylthio, arylthio, aralkyloxy, aralkylthio, carboxyl, alkoxy carbonyl, oxo, and cycloalkyl. There can be optionally inserted along the alkyl chain one or more oxygen, sulfur or substituted or unsubstituted nitrogen atoms, wherein the nitrogen substituent is hydrogen, lower alkyl (also referred to herein as “alkylaminoalkyl”), or aryl.

Thus, as used herein, the term “substituted alkyl” includes alkyl groups, as defined herein, in which one or more atoms or functional groups of the alkyl group are replaced with another atom or functional group, including for example, alkyl, substituted alkyl, halogen, aryl, substituted aryl, alkoxy, hydroxyl, nitro, amino, alkylamino, dialkylamino, sulfate, and mercapto.

The term “heteroalkyl,” by itself or in combination with another term, means, unless otherwise stated, a stable straight or branched chain, or cyclic hydrocarbon group, or combinations thereof, consisting of at least one carbon atoms and at least one heteroatom selected from the group consisting of O, N, P, Si and S, and wherein the nitrogen, phosphorus, and sulfur atoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. The heteroatom(s) O, N, P and S and Si may be placed at any interior position of the heteroalkyl group or at the position at which alkyl group is attached to the remainder of the molecule. Examples include, but are not limited to, $-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_3$, $-\text{CH}_2-\text{CH}_2-\text{NH}-\text{CH}_3$, $-\text{CH}_2-\text{CH}_2-\text{N}(\text{CH}_3)-\text{CH}_3$, $-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_3$, $-\text{CH}_2-\text{CH}_2-\text{S}(\text{O})-\text{CH}_3$, $-\text{CH}_2-\text{CH}_2-\text{S}(\text{O})_2-\text{CH}_3$, $-\text{CH}=\text{CH}-\text{O}-\text{CH}_3$, $-\text{Si}(\text{CH}_3)_3$, $-\text{CH}_2-\text{CH}=\text{N}-\text{OCH}_3$, $-\text{CH}=\text{CH}-\text{N}(\text{CH}_3)-\text{CH}_3$, $\text{O}-\text{CH}_3$, $-\text{O}-\text{CH}_2-\text{CH}_3$, and $-\text{CN}$. Up to two or three heteroatoms may be consecutive, such as, for example, $-\text{CH}_2-\text{NH}-\text{OCH}_3$ and $-\text{CH}_2-\text{O}-\text{Si}(\text{CH}_3)_3$.

As described above, heteroalkyl groups, as used herein, include those groups that are attached to the remainder of the molecule through a heteroatom, such as $-\text{C}(\text{O})\text{NR}'$, $-\text{NR}'\text{R}''$, $-\text{OR}'$, $-\text{SR}$, $-\text{S}(\text{O})\text{R}$, and/or $-\text{S}(\text{O}_2)\text{R}'$. Where “heteroalkyl” is recited, followed by recitations of specific heteroalkyl groups, such as $-\text{NR}'\text{R}$ or the like, it will be understood that the terms heteroalkyl and $-\text{NR}'\text{R}''$ are not redundant or mutually exclusive. Rather, the specific heteroalkyl groups are recited to add clarity. Thus, the term “heteroalkyl” should not be interpreted herein as excluding specific

heteroalkyl groups, such as -NR'R'' or the like.

“Cyclic” and “cycloalkyl” refer to a non-aromatic mono- or multicyclic ring system of about 3 to about 10 carbon atoms, e.g., 3, 4, 5, 6, 7, 8, 9, or 10 carbon atoms. The cycloalkyl group can be optionally partially unsaturated. The cycloalkyl group also can be optionally substituted with an alkyl group substituent as defined herein, oxo, and/or alkylene. There can be optionally inserted along the cyclic alkyl chain one or more oxygen, sulfur or substituted or unsubstituted nitrogen atoms, wherein the nitrogen substituent is hydrogen, unsubstituted alkyl, substituted alkyl, aryl, or substituted aryl, thus providing a heterocyclic group. Representative monocyclic cycloalkyl rings include cyclopentyl, cyclohexyl, and cycloheptyl. Multicyclic cycloalkyl rings include adamantyl, octahydronaphthyl, decalin, camphor, camphane, and noradamantyl, and fused ring systems, such as dihydro- and tetrahydronaphthalene, and the like.

The term “cycloalkylalkyl,” as used herein, refers to a cycloalkyl group as defined hereinabove, which is attached to the parent molecular moiety through an alkyl group, also as defined above. Examples of cycloalkylalkyl groups include cyclopropylmethyl and cyclopentylethyl.

The terms “cycloheteroalkyl” or “heterocycloalkyl” refer to a non-aromatic ring system, unsaturated or partially unsaturated ring system, such as a 3- to 10-member substituted or unsubstituted cycloalkyl ring system, including one or more heteroatoms, which can be the same or different, and are selected from the group consisting of nitrogen (N), oxygen (O), sulfur (S), phosphorus (P), and silicon (Si), and optionally can include one or more double bonds.

The cycloheteroalkyl ring can be optionally fused to or otherwise attached to other cycloheteroalkyl rings and/or non-aromatic hydrocarbon rings. Heterocyclic rings include those having from one to three heteroatoms independently selected from oxygen, sulfur, and nitrogen, in which the nitrogen and sulfur heteroatoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. In certain embodiments, the term heterocyclic refers to a non-aromatic 5-, 6-, or 7-membered ring or a polycyclic group wherein at least one ring atom is a heteroatom selected from O, S, and N (wherein the nitrogen and sulfur heteroatoms may be optionally oxidized), including, but not limited to, a bi- or tri-cyclic group, comprising fused six-membered rings having between one and three heteroatoms independently selected from the oxygen, sulfur, and nitrogen, wherein (i) each 5-membered ring has

0 to 2 double bonds, each 6-membered ring has 0 to 2 double bonds, and each 7-membered ring has 0 to 3 double bonds, (ii) the nitrogen and sulfur heteroatoms may be optionally oxidized, (iii) the nitrogen heteroatom may optionally be quaternized, and (iv) any of the above heterocyclic rings may be fused to an aryl or heteroaryl ring.

- 5 Representative cycloheteroalkyl ring systems include, but are not limited to pyrrolidinyl, pyrrolinyl, imidazolidinyl, imidazoliny, pyrazolidinyl, pyrazolinyl, piperidyl, piperazinyl, indolinyl, quinuclidinyl, morpholinyl, thiomorpholinyl, thiadiazinanyl, tetrahydrofuranyl, and the like.

- The terms "cycloalkyl" and "heterocycloalkyl," by themselves or in combination with other terms, represent, unless otherwise stated, cyclic versions of "alkyl" and "heteroalkyl," respectively. Additionally, for heterocycloalkyl, a heteroatom can occupy the position at which the heterocycle is attached to the remainder of the molecule. Examples of cycloalkyl include, but are not limited to, cyclopentyl, cyclohexyl, 1-cyclohexenyl, 3-cyclohexenyl, cycloheptyl, and the like.
- 15 Examples of heterocycloalkyl include, but are not limited to, 1-(1,2,5,6-tetrahydropyridyl), 1-piperidinyl, 2-piperidinyl, 3-piperidinyl, 4-morpholinyl, 3-morpholinyl, tetrahydrofuran-2-yl, tetrahydrofuran-3-yl, tetrahydrothien-2-yl, tetrahydrothien-3-yl, 1-piperazinyl, 2-piperazinyl, and the like. The terms "cycloalkylene" and "heterocycloalkylene" refer to the divalent derivatives of cycloalkyl and heterocycloalkyl, respectively.
- 20

- An unsaturated alkyl group is one having one or more double bonds or triple bonds. Examples of unsaturated alkyl groups include, but are not limited to, vinyl, 2-propenyl, crotyl, 2-isopentenyl, 2-(butadienyl), 2,4-pentadienyl, 3-(1,4-pentadienyl), ethynyl, 1- and 3-propynyl, 3-butylnyl, and the higher homologs and isomers. Alkyl groups which are limited to hydrocarbon groups are termed "homoalkyl."
- 25

- More particularly, the term "alkenyl" as used herein refers to a monovalent group derived from a C₁₋₂₀ inclusive straight or branched hydrocarbon moiety having at least one carbon-carbon double bond by the removal of a single hydrogen molecule. Alkenyl groups include, for example, ethenyl (i.e., vinyl), propenyl, butenyl, 1-methyl-2-buten-1-yl, pentenyl, hexenyl, octenyl, allenyl, and butadienyl.
- 30

The term "cycloalkenyl" as used herein refers to a cyclic hydrocarbon containing at least one carbon-carbon double bond. Examples of cycloalkenyl groups include cyclopropenyl, cyclobutenyl, cyclopentenyl, cyclopentadiene, cyclohexenyl, 1,3-cyclohexadiene, cycloheptenyl, cycloheptatrienyl, and cyclooctenyl.

The term “alkynyl” as used herein refers to a monovalent group derived from a straight or branched C₁₋₂₀ hydrocarbon of a designed number of carbon atoms containing at least one carbon-carbon triple bond. Examples of “alkynyl” include ethynyl, 2-propynyl (propargyl), 1-propynyl, pentynyl, hexynyl, and heptynyl groups,
 5 and the like.

The term “alkylene” by itself or a part of another substituent refers to a straight or branched bivalent aliphatic hydrocarbon group derived from an alkyl group having from 1 to about 20 carbon atoms, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 carbon atoms. The alkylene group can be straight,
 10 branched or cyclic. The alkylene group also can be optionally unsaturated and/or substituted with one or more “alkyl group substituents.” There can be optionally inserted along the alkylene group one or more oxygen, sulfur or substituted or unsubstituted nitrogen atoms (also referred to herein as “alkylaminoalkyl”), wherein the nitrogen substituent is alkyl as previously described. Exemplary alkylene groups
 15 include methylene (–CH₂–); ethylene (–CH₂–CH₂–); propylene (–(CH₂)₃–); cyclohexylene (–C₆H₁₀–); –CH=CH–CH=CH–; –CH=CH–CH₂–; –CH₂CH₂CH₂CH₂–, –CH₂CH=CHCH₂–, –CH₂CsCCH₂–, –CH₂CH₂CH(CH₂CH₂CH₃)CH₂–, –(CH₂)_q–N(R)–(CH₂)_r–, wherein each of q and r is independently an integer from 0 to about 20, e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20,
 20 and R is hydrogen or lower alkyl; methylenedioxy (–O–CH₂–O–); and ethylenedioxy (–O–(CH₂)₂–O–). An alkylene group can have about 2 to about 3 carbon atoms and can further have 6-20 carbons. Typically, an alkyl (or alkylene) group will have from 1 to 24 carbon atoms, with those groups having 10 or fewer carbon atoms being some embodiments of the present disclosure. A “lower alkyl” or
 25 “lower alkylene” is a shorter chain alkyl or alkylene group, generally having eight or fewer carbon atoms.

The term “heteroalkylene” by itself or as part of another substituent means a divalent group derived from heteroalkyl, as exemplified, but not limited by, –CH₂–CH₂–S–CH₂–CH₂– and –CH₂–S–CH₂–CH₂–NH–CH₂–. For heteroalkylene groups,
 30 heteroatoms also can occupy either or both of the chain termini (e.g., alkyleneoxo, alkylenedioxo, alkyleneamino, alkylenediamino, and the like). Still further, for alkylene and heteroalkylene linking groups, no orientation of the linking group is implied by the direction in which the formula of the linking group is written. For example, the formula –C(O)OR’– represents both –C(O)OR’– and –R’OC(O)–.

The term "aryl" means, unless otherwise stated, an aromatic hydrocarbon substituent that can be a single ring or multiple rings (such as from 1 to 3 rings), which are fused together or linked covalently. Aryls include phenyl (C₆), naphthyl (C₁₀), and biphenyl (C₁₂).

5 The term "heteroaryl" refers to aryl groups (or rings) that contain from one to four heteroatoms (in each separate ring in the case of multiple rings) selected from N, O, and S, wherein the nitrogen and sulfur atoms are optionally oxidized, and the nitrogen atom(s) are optionally quaternized. A heteroaryl group can be attached to the remainder of the molecule through a carbon or heteroatom. Non-limiting examples of
10 aryl and heteroaryl groups include phenyl, 1-naphthyl, 2-naphthyl, 4-biphenyl, 1-pyrrolyl, 2-pyrrolyl, 3-pyrrolyl, 3-pyrazolyl, 2-imidazolyl, 4-imidazolyl, pyrazinyl, 2-oxazolyl, 4-oxazolyl, 2-phenyl-4-oxazolyl, 5-oxazolyl, 3-isoxazolyl, 4-isoxazolyl, 5-isoxazolyl, 2-thiazolyl, 4-thiazolyl, 5-thiazolyl, 2-furyl, 3-furyl, 2-thienyl, 3-thienyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrimidyl, 4-pyrimidyl, 5-benzothiazolyl, purinyl,
15 2-benzimidazolyl, 5-indolyl, 1-isoquinolyl, 5-isoquinolyl, 2-quinoxalyl, 5-quinoxalyl, 3-quinolyl, and 6-quinolyl. Substituents for each of above noted aryl and heteroaryl ring systems are selected from the group of acceptable substituents described below. The terms "arylene" and "heteroarylene" refer to the divalent forms of aryl and heteroaryl, respectively.

20 In further embodiments, the term "heteroaryl" refers to a C₅-C₂₀ aromatic ring wherein at least one carbon atom is replaced by a heteroatom selected from O, S, N, optionally substituted by at least one substituent selected from the group consisting of C₁-C₆ alkyl, hydroxy, C₁-C₄ alkoxy, mercapto, C₁-C₄ alkylthio, amino, -NH(C₁-C₆ alkyl), -N(C₁-C₆ alkyl)₂, -O(C₆-C₁₂ aryl), -N(C₆-C₁₂ aryl)₂, -NH(C₆-C₁₂ aryl), -S(C₆-
25 C₁₂ aryl), halogen, -CF₃, -SO₃H, -COOH, -COO(C₁-C₈ alkyl), -SO₂NH₂, -SO₂NH(C₁-C₆ alkyl or C₆-C₁₂ aryl), -CN, -NO₃, -C(O)(C₁-C₈ alkyl), -C(O)(C₆-C₁₂ aryl), -N(C₁-C₆ alkyl or H)C(O)(C₁-C₆ alkyl or H), -C(O)N(C₁-C₆ alkyl or H)₂.

Exemplary heteroaryls include, but are not limited to, pyridyl, pyrimidinyl, pyrazinyl, furanyl, thienyl, pyrazolyl, oxazolyl, thiazolyl, isothiazolyl, isoxazolyl,
30 pyrrolyl, imidazolyl, indolyl, indolinolyl, and imidazopyridazinyl.

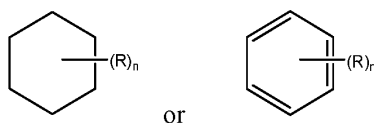
In further embodiments, the term "aryl" also can refer to C₆-C₁₄ aryl, optionally substituted by at least one substituent selected from the group consisting of C₁-C₆ alkyl, hydroxy, C₁-C₄ alkoxy, mercapto, C₁-C₄ alkylthio, amino, -NH(C₁-C₆ alkyl), -N(C₁-C₆ alkyl)₂, -O(C₆-C₁₂ aryl), -N(C₆-C₁₂ aryl)₂, -NH(C₆-C₁₂ aryl), -S(C₆-

C₁₂ aryl), halogen, -CF₃, -SO₃H, -COOH, -COO(C₁-C₈ alkyl), -SO₂NH₂, -SO₂NH(C₁-C₆ alkyl or C₆-C₁₂ aryl), -CN, -NO₃, -C(O)(C₁-C₈ alkyl), -C(O)(C₆-C₁₂ aryl), -N(C₁-C₆ alkyl or H)C(O)(C₁-C₆ alkyl or H), -C(O)N(C₁-C₆ alkyl or H)₂.

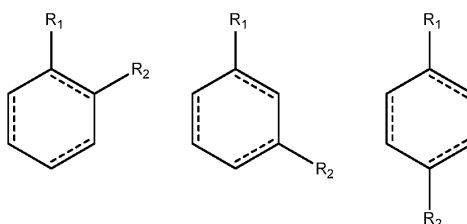
For brevity, the term “aryl” when used in combination with other terms (e.g.,
 5 aryloxy, arylthioxy, arylalkyl) includes both aryl and heteroaryl rings as defined
 above. Thus, the terms “arylalkyl” and “heteroarylalkyl” are meant to include those
 groups in which an aryl or heteroaryl group is attached to an alkyl group (e.g., benzyl,
 phenethyl, pyridylmethyl, furylmethyl, and the like) including those alkyl groups in
 which a carbon atom (e.g., a methylene group) has been replaced by, for example, an
 10 oxygen atom (e.g., phenoxy methyl, 2-pyridyloxy methyl, 3-(1-naphthyloxy)propyl, and
 the like). However, the term “haloaryl,” as used herein is meant to cover only aryls
 substituted with one or more halogens.

Where a heteroalkyl, heterocycloalkyl, or heteroaryl includes a specific
 number of members (e.g., “3 to 7 membered”), the term “member” refers to a carbon
 15 or heteroatom.

Further, a structure represented generally by the formula:



as used herein refers to a ring structure, for example, but not limited to a 3-carbon, a
 4-carbon, a 5-carbon, a 6-carbon, a 7-carbon, and the like, aliphatic and/or aromatic
 20 cyclic compound, including a saturated ring structure, a partially saturated ring
 structure, and an unsaturated ring structure, comprising a substituent R group, wherein
 the R group can be present or absent, and when present, one or more R groups can
 each be substituted on one or more available carbon atoms of the ring structure. The
 presence or absence of the R group and number of R groups is determined by the
 25 value of the variable “n,” which is an integer generally having a value ranging from 0
 to the number of carbon atoms on the ring available for substitution. Each R group, if
 more than one, is substituted on an available carbon of the ring structure rather than
 on another R group. For example, the structure above where n is 0 to 2 would
 comprise compound groups including, but not limited to:



and the like.

A dashed line representing a bond in a cyclic ring structure indicates that the bond can be either present or absent in the ring. That is, a dashed line representing a bond in a cyclic ring structure indicates that the ring structure is selected from the group consisting of a saturated ring structure, a partially saturated ring structure, and an unsaturated ring structure.

The symbol () denotes the point of attachment of a moiety to the remainder of the molecule.

- 10 When a named atom of an aromatic ring or a heterocyclic aromatic ring is defined as being “absent,” the named atom is replaced by a direct bond.

- Each of above terms (e.g., “alkyl,” “heteroalkyl,” “cycloalkyl,” and “heterocycloalkyl,” “aryl,” “heteroaryl,” “phosphonate,” and “sulfonate” as well as their divalent derivatives) are meant to include both substituted and unsubstituted forms of the indicated group. Optional substituents for each type of group are provided below.

- Substituents for alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl monovalent and divalent derivative groups (including those groups often referred to as alkylene, alkenyl, heteroalkylene, heteroalkenyl, alkynyl, cycloalkyl, heterocycloalkyl, cycloalkenyl, and heterocycloalkenyl) can be one or more of a variety of groups selected from, but not limited to: -OR', =O, =NR', =N-OR', -NR'R'', -SR', -halogen, -SiR'R''R''', -OC(O)R', -C(O)R', -CO₂R', -C(O)NR'R'', -OC(O)NR'R'', -NR''C(O)R', -NR'-C(O)NR''R''', -NR''C(O)OR', -NR-C(NR'R'')=NR'', -S(O)R', -S(O)₂R', -S(O)₂NR'R'', -NRSO₂R', -CN and -NO₂ in a number ranging from zero to (2m'+1), where m' is the total number of carbon atoms in such groups. R', R'', R''' and R'''' each may independently refer to hydrogen, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl (e.g., aryl substituted with 1-3 halogens), substituted or unsubstituted alkyl, alkoxy or thioalkoxy groups, or arylalkyl

groups. As used herein, an “alkoxy” group is an alkyl attached to the remainder of the molecule through a divalent oxygen. When a compound of the disclosure includes more than one R group, for example, each of the R groups is independently selected as are each R', R'', R''' and R'''' groups when more than one of these groups is present. When R' and R'' are attached to the same nitrogen atom, they can be combined with the nitrogen atom to form a 4-, 5-, 6-, or 7- membered ring. For example, -NR'R'' is meant to include, but not be limited to, 1- pyrrolidinyl and 4-morpholinyl. From the above discussion of substituents, one of skill in the art will understand that the term “alkyl” is meant to include groups including carbon atoms bound to groups other than hydrogen groups, such as haloalkyl (e.g., -CF₃ and -CH₂CF₃) and acyl (e.g., -C(O)CH₃, -C(O)CF₃, -C(O)CH₂OCH₃, and the like).

Similar to the substituents described for alkyl groups above, exemplary substituents for aryl and heteroaryl groups (as well as their divalent derivatives) are varied and are selected from, for example: halogen, -OR', -NR'R'', -SR', -SiR'R''R''', -OC(O)R', -C(O)R', -CO₂R', -C(O)NR'R'', -OC(O)NR'R'', -NR''C(O)R', -NR'-C(O)NR''R''', -NR''C(O)OR', -NR-C(NR'R''R''')=NR''', -NR-C(NR'R''R''')=NR'''-S(O)R', -S(O)₂R', -S(O)₂NR'R'', -NRSO₂R', -CN and -NO₂, -R', -N₃, -CH(Ph)₂, fluoro(C₁-C₄)alkoxo, and fluoro(C₁-C₄)alkyl, in a number ranging from zero to the total number of open valences on aromatic ring system; and where R', R'', R''' and R'''' may be independently selected from hydrogen, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl and substituted or unsubstituted heteroaryl. When a compound of the disclosure includes more than one R group, for example, each of the R groups is independently selected as are each R', R'', R''' and R'''' groups when more than one of these groups is present.

Two of the substituents on adjacent atoms of aryl or heteroaryl ring may optionally form a ring of the formula -T-C(O)-(CRR')_q-U-, wherein T and U are independently -NR-, -O-, -CRR'- or a single bond, and q is an integer of from 0 to 3. Alternatively, two of the substituents on adjacent atoms of aryl or heteroaryl ring may optionally be replaced with a substituent of the formula -A-(CH₂)_r-B-, wherein A and B are independently -CRR'-, -O-, -NR-, -S-, -S(O)-, -S(O)₂-, -S(O)₂NR'- or a single bond, and r is an integer of from 1 to 4.

One of the single bonds of the new ring so formed may optionally be replaced

with a double bond. Alternatively, two of the substituents on adjacent atoms of aryl or heteroaryl ring may optionally be replaced with a substituent of the formula $-(CRR')_s-X'-(C''R'')_d-$, where s and d are independently integers of from 0 to 3, and X' is $-O-$, $-NR'-$, $-S-$, $-S(O)-$, $-S(O)_2-$, or $-S(O)_2NR'-$. The substituents R , R' , R'' and R''' may be independently selected from hydrogen, substituted or unsubstituted alkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, and substituted or unsubstituted heteroaryl.

As used herein, the term “acyl” refers to an organic acid group wherein the $-OH$ of the carboxyl group has been replaced with another substituent and has the general formula $RC(=O)-$, wherein R is an alkyl, alkenyl, alkynyl, aryl, carbocyclic, heterocyclic, or aromatic heterocyclic group as defined herein). As such, the term “acyl” specifically includes arylacyl groups, such as a 2-(furan-2-yl)acetyl- and a 2-phenylacetyl group. Specific examples of acyl groups include acetyl and benzoyl. Acyl groups also are intended to include amides, $-RC(=O)NR'$, esters, $-RC(=O)OR'$, ketones, $-RC(=O)R'$, and aldehydes, $-RC(=O)H$.

The terms “alkoxyl” or “alkoxy” are used interchangeably herein and refer to a saturated (i.e., alkyl-O-) or unsaturated (i.e., alkenyl-O- and alkynyl-O-) group attached to the parent molecular moiety through an oxygen atom, wherein the terms “alkyl,” “alkenyl,” and “alkynyl” are as previously described and can include C_{1-20} inclusive, linear, branched, or cyclic, saturated or unsaturated oxo-hydrocarbon chains, including, for example, methoxyl, ethoxyl, propoxyl, isopropoxyl, *n*-butoxyl, *sec*-butoxyl, *tert*-butoxyl, and *n*-pentoxyl, neopentoxyl, *n*-hexoxyl, and the like.

The term “alkoxyalkyl” as used herein refers to an alkyl-O-alkyl ether, for example, a methoxyethyl or an ethoxymethyl group.

“Aryloxyl” refers to an aryl-O- group wherein the aryl group is as previously described, including a substituted aryl. The term “aryloxyl” as used herein can refer to phenyloxyl or hexyloxyl, and alkyl, substituted alkyl, halo, or alkoxy substituted phenyloxyl or hexyloxyl.

“Aralkyl” refers to an aryl-alkyl-group wherein aryl and alkyl are as previously described, and included substituted aryl and substituted alkyl. Exemplary aralkyl groups include benzyl, phenylethyl, and naphthylmethyl.

“Aralkyloxyl” refers to an aralkyl-O- group wherein the aralkyl group is as previously described. An exemplary aralkyloxyl group is benzyloxyl, i.e., $C_6H_5-CH_2-O-$. An aralkyloxyl group can optionally be substituted.

“Alkoxy carbonyl” refers to an alkyl-O-C(=O)- group. Exemplary alkoxy carbonyl groups include methoxy carbonyl, ethoxy carbonyl, butyloxy carbonyl, and *tert*-butyloxy carbonyl.

5 “Aryloxy carbonyl” refers to an aryl-O-C(=O)- group. Exemplary aryloxy carbonyl groups include phenoxy- and naphthoxy-carbonyl.

“Aralkoxy carbonyl” refers to an aralkyl-O-C(=O)- group. An exemplary aralkoxy carbonyl group is benzyloxy carbonyl.

“Carbamoyl” refers to an amide group of the formula -C(=O)NH₂.

10 “Alkyl carbamoyl” refers to a R'RN-C(=O)- group wherein one of R and R' is hydrogen and the other of R and R' is alkyl and/or substituted alkyl as previously described. “Dialkyl carbamoyl” refers to a R'RN-C(=O)- group wherein each of R and R' is independently alkyl and/or substituted alkyl as previously described.

The term carbonyldioxy, as used herein, refers to a carbonate group of the formula -O-C(=O)-OR.

15 “Acyloxy” refers to an acyl-O- group wherein acyl is as previously described.

The term “amino” refers to the -NH₂ group and also refers to a nitrogen containing group as is known in the art derived from ammonia by the replacement of one or more hydrogen radicals by organic radicals. For example, the terms “acylamino” and “alkylamino” refer to specific N-substituted organic radicals with
20 acyl and alkyl substituent groups respectively.

An “aminoalkyl” as used herein refers to an amino group covalently bound to an alkylene linker. More particularly, the terms alkylamino, dialkylamino, and trialkylamino as used herein refer to one, two, or three, respectively, alkyl groups, as previously defined, attached to the parent molecular moiety through a nitrogen atom.
25 The term alkylamino refers to a group having the structure -NHR' wherein R' is an alkyl group, as previously defined; whereas the term dialkylamino refers to a group having the structure -NR'R'', wherein R' and R'' are each independently selected from the group consisting of alkyl groups. The term trialkylamino refers to a group having the structure -NR'R''R''', wherein R', R'', and R''' are each independently
30 selected from the group consisting of alkyl groups. Additionally, R', R'', and/or R''' taken together may optionally be -(CH₂)_k- where k is an integer from 2 to 6. Examples include, but are not limited to, methylamino, dimethylamino, ethylamino, diethylamino, diethylaminocarbonyl, methylethylamino, isopropylamino, piperidino, trimethylamino, and propylamino.

The amino group is -NR'R'', wherein R' and R'' are typically selected from hydrogen, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, or substituted or unsubstituted heteroaryl.

5 The terms alkylthioether and thioalkoxyl refer to a saturated (i.e., alkyl-S-) or unsaturated (i.e., alkenyl-S- and alkynyl-S-) group attached to the parent molecular moiety through a sulfur atom. Examples of thioalkoxyl moieties include, but are not limited to, methylthio, ethylthio, propylthio, isopropylthio, *n*-butylthio, and the like.

 “Acylamino” refers to an acyl-NH- group wherein acyl is as previously
10 described. “Aroylamino” refers to an aroyl-NH- group wherein aroyl is as previously described.

The term “carbonyl” refers to the -C(=O)- group, and can include an aldehyde group represented by the general formula R-C(=O)H.

 The term “carboxyl” refers to the -COOH group. Such groups also are
15 referred to herein as a “carboxylic acid” moiety.

 The terms “halo,” “halide,” or “halogen” as used herein refer to fluoro, chloro, bromo, and iodo groups. Additionally, terms, such as “haloalkyl,” are meant to include monohaloalkyl and polyhaloalkyl. For example, the term “halo(C₁-C₄)alkyl” is mean to include, but not be limited to, trifluoromethyl, 2,2,2-trifluoroethyl, 4-
20 chlorobutyl, 3-bromopropyl, and the like.

 The term “hydroxyl” refers to the -OH group.

 The term “hydroxyalkyl” refers to an alkyl group substituted with an -OH group.

 The term “mercapto” refers to the -SH group.

25 The term “oxo” as used herein means an oxygen atom that is double bonded to a carbon atom or to another element.

 The term “nitro” refers to the -NO₂ group.

 The term “thio” refers to a compound described previously herein wherein a carbon or oxygen atom is replaced by a sulfur atom.

30 The term “sulfate” refers to the -SO₄ group.

 The term thiohydroxyl or thiol, as used herein, refers to a group of the formula -SH.

 More particularly, the term “sulfide” refers to compound having a group of the formula -SR.

The term “sulfone” refers to compound having a sulfonyl group $-S(O_2)R$.

The term “sulfoxide” refers to a compound having a sulfinyl group $-S(O)R$.

The term ureido refers to a urea group of the formula $-NH-CO-NH_2$.

Throughout the specification and claims, a given chemical formula or name
5 shall encompass all tautomers, congeners, and optical- and stereoisomers, as well as
racemic mixtures where such isomers and mixtures exist.

Certain compounds of the present disclosure may possess asymmetric carbon
atoms (optical or chiral centers) or double bonds; the enantiomers, racemates,
diastereomers, tautomers, geometric isomers, stereoisometric forms that may be
10 defined, in terms of absolute stereochemistry, as (R)- or (S)- or, as D- or L- for amino
acids, and individual isomers are encompassed within the scope of the present
disclosure. The compounds of the present disclosure do not include those which are
known in art to be too unstable to synthesize and/or isolate. The present disclosure is
meant to include compounds in racemic, scalemic, and optically pure forms.

15 Optically active (R)- and (S)-, or D- and L-isomers may be prepared using chiral
synthons or chiral reagents, or resolved using conventional techniques. When the
compounds described herein contain olefinic bonds or other centers of geometric
asymmetry, and unless specified otherwise, it is intended that the compounds include
both *E* and *Z* geometric isomers.

20 Unless otherwise stated, structures depicted herein are also meant to include
all stereochemical forms of the structure; i.e., the R and S configurations for each
asymmetric center. Therefore, single stereochemical isomers as well as enantiomeric
and diastereomeric mixtures of the present compounds are within the scope of the
disclosure.

25 It will be apparent to one skilled in the art that certain compounds of this
disclosure may exist in tautomeric forms, all such tautomeric forms of the compounds
being within the scope of the disclosure. The term “tautomer,” as used herein, refers
to one of two or more structural isomers which exist in equilibrium and which are
readily converted from one isomeric form to another.

30 Unless otherwise stated, structures depicted herein are also meant to include
compounds which differ only in the presence of one or more isotopically enriched
atoms. For example, compounds having the present structures with the replacement
of a hydrogen by a deuterium or tritium, or the replacement of a carbon by ^{13}C - or
 ^{14}C -enriched carbon are within the scope of this disclosure.

The compounds of the present disclosure may also contain unnatural proportions of atomic isotopes at one or more of atoms that constitute such compounds. For example, the compounds may be radiolabeled with radioactive isotopes, such as for example tritium (^3H), iodine-125 (^{125}I) or carbon-14 (^{14}C). All isotopic variations of the compounds of the present disclosure, whether radioactive or not, are encompassed within the scope of the present disclosure.

The compounds of the present disclosure may exist as salts. The present disclosure includes such salts. Examples of applicable salt forms include hydrochlorides, hydrobromides, sulfates, methanesulfonates, nitrates, maleates, acetates, citrates, fumarates, tartrates (e.g., (+)-tartrates, (-)-tartrates or mixtures thereof including racemic mixtures, succinates, benzoates and salts with amino acids, such as glutamic acid. These salts may be prepared by methods known to those skilled in art. Also included are base addition salts, such as sodium, potassium, calcium, ammonium, organic amino, or magnesium salt, or a similar salt. When compounds of the present disclosure contain relatively basic functionalities, acid addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired acid, either neat or in a suitable inert solvent or by ion exchange. Examples of acceptable acid addition salts include those derived from inorganic acids like hydrochloric, hydrobromic, nitric, carbonic, monohydrogencarbonic, phosphoric, monohydrogenphosphoric, dihydrogenphosphoric, sulfuric, monohydrogensulfuric, hydriodic, or phosphorous acids and the like, as well as the salts derived organic acids like acetic, propionic, isobutyric, maleic, malonic, benzoic, succinic, suberic, fumaric, lactic, mandelic, phthalic, benzenesulfonic, p-tolylsulfonic, citric, tartaric, methanesulfonic, and the like. Also included are salts of amino acids, such as arginate and the like, and salts of organic acids like glucuronic or galactunoric acids and the like. Certain specific compounds of the present disclosure contain both basic and acidic functionalities that allow the compounds to be converted into either base or acid addition salts.

The neutral forms of the compounds may be regenerated by contacting the salt with a base or acid and isolating the parent compound in the conventional manner. The parent form of the compound differs from the various salt forms in certain physical properties, such as solubility in polar solvents.

Certain compounds of the present disclosure can exist in unsolvated forms as well as solvated forms, including hydrated forms. In general, the solvated forms are

equivalent to unsolvated forms and are encompassed within the scope of the present disclosure. Certain compounds of the present disclosure may exist in multiple crystalline or amorphous forms. In general, all physical forms are equivalent for the uses contemplated by the present disclosure and are intended to be within the scope of the present disclosure.

In addition to salt forms, the present disclosure provides compounds, which are in a prodrug form. Prodrugs of the compounds described herein are those compounds that readily undergo chemical changes under physiological conditions to provide the compounds of the present disclosure. Additionally, prodrugs can be converted to the compounds of the present disclosure by chemical or biochemical methods in an ex vivo environment. For example, prodrugs can be slowly converted to the compounds of the present disclosure when placed in a transdermal patch reservoir with a suitable enzyme or chemical reagent.

Following long-standing patent law convention, the terms “a,” “an,” and “the” refer to “one or more” when used in this application, including the claims. Thus, for example, reference to “a subject” includes a plurality of subjects, unless the context clearly is to the contrary (e.g., a plurality of subjects), and so forth.

Throughout this specification and the claims, the terms “comprise,” “comprises,” and “comprising” are used in a non-exclusive sense, except where the context requires otherwise. Likewise, the term “include” and its grammatical variants are intended to be non-limiting, such that recitation of items in a list is not to the exclusion of other like items that can be substituted or added to the listed items.

For the purposes of this specification and appended claims, unless otherwise indicated, all numbers expressing amounts, sizes, dimensions, proportions, shapes, formulations, parameters, percentages, quantities, characteristics, and other numerical values used in the specification and claims, are to be understood as being modified in all instances by the term “about” even though the term “about” may not expressly appear with the value, amount or range. Accordingly, unless indicated to the contrary, the numerical parameters set forth in the following specification and attached claims are not and need not be exact, but may be approximate and/or larger or smaller as desired, reflecting tolerances, conversion factors, rounding off, measurement error and the like, and other factors known to those of skill in the art depending on the desired properties sought to be obtained by the presently disclosed subject matter. For example, the term “about,” when referring to a value can be

meant to encompass variations of, in some embodiments, $\pm 100\%$ in some
embodiments $\pm 50\%$, in some embodiments $\pm 20\%$, in some embodiments $\pm 10\%$, in
some embodiments $\pm 5\%$, in some embodiments $\pm 1\%$, in some embodiments $\pm 0.5\%$,
and in some embodiments $\pm 0.1\%$ from the specified amount, as such variations are
5 appropriate to perform the disclosed methods or employ the disclosed compositions.

Further, the term “about” when used in connection with one or more numbers
or numerical ranges, should be understood to refer to all such numbers, including all
numbers in a range and modifies that range by extending the boundaries above and
below the numerical values set forth. The recitation of numerical ranges by endpoints
10 includes all numbers, e.g., whole integers, including fractions thereof, subsumed
within that range (for example, the recitation of 1 to 5 includes 1, 2, 3, 4, and 5, as
well as fractions thereof, e.g., 1.5, 2.25, 3.75, 4.1, and the like) and any range within
that range.

15 EXAMPLES

The following Examples have been included to provide guidance to one of
ordinary skill in the art for practicing representative embodiments of the presently
disclosed subject matter. In light of the present disclosure and the general level of
skill in the art, those of skill can appreciate that the following Examples are intended
20 to be exemplary only and that numerous changes, modifications, and alterations can
be employed without departing from the scope of the presently disclosed subject
matter. The synthetic descriptions and specific examples that follow are only
intended for the purposes of illustration, and are not to be construed as limiting in any
manner to make compounds of the disclosure by other methods.

25

EXAMPLE 1

Methods

General Procedures: The commercially available HPLC grade methanol, catalysts
and reagent grade materials were used as received. TLC was performed on Silica gel
30 60 F254-coated aluminum sheets (Merck) and spots were detected by the solution of
 $\text{Ce}(\text{SO}_4)_2 \cdot 4\text{H}_2\text{O}$ (1%) and $\text{H}_3\text{P}(\text{Mo}_3\text{O}_{10})_4$ (2%) in sulfuric acid (10%). Flash
chromatography was performed on Silica gel 60 (0.040-0.063 mm, Fluka) or on
Biotage® KP-C18-HS or KP-Sil® SNAP cartridges using the Isolera One HPFC

system (Biotage, Inc.). All chemicals were purchased from Sigma-Aldrich and were used without further purification. The ^1H NMR spectra were measured at 400.1 MHz, 500.1 MHz or 600.1 MHz, ^{13}C NMR spectra at 100.8 MHz, 125.7 MHz or 150.9 MHz. For standardization of ^1H NMR spectra the internal signal of TMS (δ 0.0, CDCl_3) or residual signals of solvents (δ 7.26 for CDCl_3 , δ 2.05 for CD_3COCD_3 and δ 3.31 for CD_3OD) were used. In the case of ^{13}C spectra the residual signals of solvents (δ 77.00 for CDCl_3 , δ 29.84 and δ 206.26 for CD_3COCD_3 and δ 49.00 for CD_3OD) were used. The chemical shifts are given in δ -scale, the coupling constants J are given in Hz. The ESI mass spectra were recorded using ZQ micromass mass spectrometer (Waters) equipped with an ESCi multimode ion source and controlled by MassLynx software. Alternatively, the low resolution ESI mass spectra were recorded using a quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-ToF micro, Waters) and high resolution ESI mass spectra using a hybrid FT mass spectrometer combining a linear ion trap MS and the Orbitrap mass analyzer (LTQ Orbitrap XL, Thermo Fisher Scientific). The conditions were optimized for suitable ionization in the ESI Orbitrap source (sheath gas flow rate 35 a.u., aux gas flow rate 10 a.u. of nitrogen, source voltage 4.3 kV, capillary voltage 40 V, capillary temperature 275 $^\circ\text{C}$, tube lens voltage 155 V). The samples were dissolved in methanol and applied by direct injection. Optical rotations were measured in CHCl_3 or DMF using an Autopol IV instrument (Rudolph Research Analytical). The IR spectra were measured in CHCl_3 or KBr.

Mice Efficacy Studies. All mouse efficacy studies were conducted according to protocol #MO13M69 approved by the Animal Care and Use Committee at Johns Hopkins University. Female athymic (RH-Foxn1nu mice) mice between 25 and 30g were obtained (Harlan Sprague Dawley Inc, Indianapolis, Indiana), and maintained on a 12 hour light-dark cycle with *ad libitum* access to food and water. U87 human glioma cells were injected s.c. (5×10^6 cells in 100 μl of PBS) in four separate locations on the flanks of each mouse. When tumors grew to a mean volume of around 200 mm^3 , mice were randomized into either vehicle (HEPES-buffered saline, i.p.) or DON (1; 0.8 mg/kg, i.p.). In one cohort, mice were administered a single dose of the appropriate solution two hours after which glutamine levels were quantified in the tumor as described previously (Le et al., 2012). In brief, tumors were harvested, snap frozen, and homogenized in liquid N_2 then subjected to metabolite extraction

using methanol and DI water. Quantification was performed using Agilent 6520 Quadrupole–Time-of-Flight (Q-TOF) mass spectrometer with Agilent 1290 HPLC and using Agilent Mass Hunter and Agilent Qualitative and Quantitative Analysis Software packages. Glutamine content was averaged by group for each individual tumor (n=3-4/group), depicted as relative intensity, and analyzed by one-tailed t test. In a second cohort, efficacy experiments were conducted. Mice were injected once daily for six days; tumor volumes were measured using digital calipers and calculated according to the formula: [volume= (largest tumor dimension) x (smallest tumor dimension)² x 0.52] at 2, 4, and 6 days after the onset of treatment. Each individual tumor (n=8-10/group) was normalized to its pretreatment volume, averaged and analyzed by repeated measures two-way analysis of variance (ANOVA). If significant, a Bonferroni post hoc test was subsequently applied. Significance was defined as $p < .05$.

In Vitro Stability Studies: The stock solution for most prodrugs was prepared as a 10 mM solution in DMSO to carry out the *in vitro* studies.

The chemical stability of prodrugs was evaluated using simulated gastric fluid (SGF; pH 1.2) and phosphate buffered saline (PBS; pH 7.4). Briefly, prodrugs were spiked (10 μ M) in respective solutions and incubated at 37 °C for 1h. At predetermined time points (0, 30 and 60 min), aliquots of 100 μ L were removed and diluted with 100 μ L of water. Prodrug disappearance was monitored using the developed liquid chromatography and tandem mass spectrometry (LC/MS/MS) method described below.

For metabolic stability, plasma (dog, human, monkey, mouse and pig and human) was used. For stability, prodrugs (10 μ M) were spiked in each matrix and incubated in an orbital shaker at 37 °C. At predetermined times (0, 30 and 60 min), 100 μ L aliquots of the mixture in triplicate were removed and the reaction quenched by addition of three times the volume of ice cold acetonitrile spiked with the internal standard (losartan 0.5 μ M). The samples were vortexed for 30 s and centrifuged 12000 g for 10 min. 50 μ L supernatant diluted with 50 μ L water was transferred to a 250 μ L polypropylene vial sealed with a Teflon cap. Prodrug disappearance was monitored over time using a liquid chromatography and tandem mass spectrometry (LC/MS/MS) method as described below.

For LC/MS/MS, prodrugs were separated with Thermo Scientific Accela UPLC system coupled to Accela open autosampler on an Agilent C18 (100x 2.1mm

id) UPLC column. The autosampler was temperature controlled and operating at 10° C. The mobile phase used for the chromatographic separation was composed of acetonitrile/water containing 0.1% formic acid and was run at a flow rate of 0.5 mL/minute for 4.5 minutes using gradient elution. The column effluent was monitored using TSQ Vantage triple-quadrupole mass-spectrometric detector, equipped with an electrospray probe set in the positive ionization mode. Samples were introduced into the ionization source through a heated nebulized probe (350 °C).

For quantification of compound remaining, disappearance of prodrugs was measured from ratio of peak areas of analyte to IS. Percentage remaining was calculated in the following manner:

$$\frac{\text{Avg. Response} * @60 \text{ min}}{\text{Avg. Response} @ 0 \text{ min}} \times 100$$

where response = [(Area of analyte)/ (Area of internal standard)]

*Average response is average of two samples at each time point.

Pharmacokinetic Studies in Mice. All pharmacokinetic studies in mice were conducted according to protocol (#MO13M113) approved by the Animal Care and Use Committee at Johns Hopkins University. C57BL/6 mice between 25 and 30g were obtained from Harlan, and maintained on a 12 hour light-dark cycle with *ad libitum* access to food and water. To evaluate the brain and plasma pharmacokinetics of DON and its prodrug **5c**, 8-12 week old C57BL/6 were administered DON (**1**; 0.8 mg/kg, p.o. in phosphate-buffered saline) and its prodrug **5c** (at 0.8 mg/kg equivalent DON (**1**), p.o. in phosphate-buffered saline with 5% EtOH and 5% Tween-80). The mice were sacrificed by pentobarbital injection at 10, 30 and 90 minutes post drug administration, and blood was collected via cardiac puncture and placed into iced EDTA coated BD microtainers. Blood samples were spun at 2,000 g for 15 minutes, and plasma was removed and stored at -80°C. Brain tissues were harvested following blood collection and immediately snap frozen in liquid nitrogen and stored at -80°C until LC/MS analysis.

Pharmacokinetic Studies in Non-human primates. All monkey studies were conducted according to protocol (#PR15M298) approved by the Animal Care and Use Committee at Johns Hopkins University. Two female pigtail monkeys (approximately 3.5 kg, non-drug naive) were adjacently housed in stainless steel cages on a social interaction rack (contains 4 cages, each 32.5" wide x 28" deep x 32"

high) maintaining temperature of 64–84 °F, humidity of 30 – 70% with alternating 14-10 hour light/dark cycle as per the USDA Animal Welfare Act (9 CFR, Parts 1, 2, and 3). Food was provided daily in amounts appropriate for the size and age of the animals and RO purified water provided *ad libitum* through an in-cage lixit valve.

- 5 Food enrichment was provided Monday through Friday. Prior to drug administration, macaques were sedated with ketamine given as an intramuscular injection prior to test article administration. Sedation was maintained through blood and cerebrospinal fluid (CSF) sample collections with ketamine at a starting rate of 15 mg/kg with additional doses of 20-30 mg during the first hour. At subsequent time points
- 10 ketamine was given at 10-15 mg/kg. DON (50mM HEPES buffered saline) and **5c** (Diastereoisomer 1), (50mM HEPES buffered saline containing 5% ethanol and 5% tween) were administered (1.6 mg/kg equivalent) to the animals at a dosing volume of 1mL/kg intravenously. CSF sample (target of 50 µL) was obtained by percutaneous puncture of the cisterna magna at 30 min post dose. Blood samples (1 mL) were
- 15 collected at 15, 30, 1, 2 4 and 6 h post dose by percutaneous puncture of a peripheral vein. Samples were processed for plasma (centrifuged at a temperature of 4°C, at 3,000xg, for 10 minutes). All samples were maintained chilled on ice throughout processing. Samples were collected in microcentrifuge tubes, flash frozen, and placed in a freezer set to maintain -80°C until LC/MS analysis.

- 20 *Bioanalysis of DON.* A highly sensitive method for analysis of DON in biological matrices (Alt, et al., 2015) has been previously published. However due to chemical lability of DON and its prodrugs, a milder derivatization method employing dabsyl chloride was developed and validated. Briefly, DON was extracted from samples (50 mg) with 250 µL methanol containing Glutamate-d5 (10 µM ISTD) by
- 25 vortexing in low retention tubes. Samples were centrifuged at 16,000 X g for 5 minutes to precipitate proteins. Supernatants (200 µL) were moved to new tube and dried at 45°C under vacuum for 1 hour. To each tube, 50 µL of 0.2 M sodium bicarbonate buffer (pH 9.0) and 100 µL of 10 mM dabsyl chloride in acetone was added. After vortexing, samples were incubated at 60°C for 15 minutes to derivatize.
- 30 Samples (2 µL) were injected and separated on an Agilent 1290 equipped with a an Agilent Eclipse plus C18 RRHD 2.1 X100 mm column over a 2.5 minute gradient from 20-95% acetonitrile + 0.1% formic acid and quantified on an Agilent 6520 QTOF mass spectrometer. Calibration curves over the range of .005–17.1 µg/mL in

plasma and CSF for DON were constructed from the peak area ratio of the analyte to the internal standard using linear regression with a weighting factor of 1/(nominal concentration). Correlation coefficient of greater than 0.99 was obtained in all analytical runs. The mean predicted relative standard deviation for back calculated concentrations of the standards and QC's for all analytes were within the range of 85 to 115%, except for the lowest concentration which was within the range of 80 to 120% with an overall accuracy and precision of 6.7% and 6.6%, respectively.

Pharmacokinetic Analysis. Mean concentration-time data were used for pharmacokinetic analysis. Non-compartmental-analysis module in WinNonlin® (version 5.3) was used to assess pharmacokinetic parameters. Peak plasma concentrations ($C_{\max}$) and time to $C_{\max}$ ($T_{\max}$) were the observed values. Area under the curve (AUC) was calculated by log-linear trapezoidal rule to the end of sample collection (AUC_{last}).

Procedure for Pharmacokinetic analysis for DON release from its prodrugs:

DON is extracted from samples (50 μL) with 250 μL methanol containing Glutamate-d5 (10 μM ISTD) by vortexing in low retention tubes. Samples are centrifuged at 16,000 X g for 5 minutes to precipitate proteins. Supernatants (200 μL) are moved to new tube and dried at 45°C under vacuum (approximately 1 hour). To each tube, 50 μL of 0.2 M sodium bicarbonate buffer (pH 9.0) and 100 μL dabsyl chloride stock is added. After vortexing, samples are incubated at 60°C for 15 minutes to derivatize. Samples (2-10 μL) are injected and separated on an Agilent 1290 equipped with a SB-AQ column over a 4 minute gradient from 20-95% acetonitrile + 0.1% formic acid and quantified on an Agilent 6520 QTOF mass spectrometer.

EXAMPLE 2

Prodrug Strategy – Masking Carboxylate Functionality

In one embodiment, DON prodrugs were designed by masking only the carboxylate functionalities using alkyl esters of DON with unprotected α -amino groups. Some of the alkyl esters of DON with an unprotected α -amino functionality, however, were found to undergo cyclization forming a 5-membered cyclic Schiff's base. The cyclization observed was pH dependent and rapid at pH 5-7. At a lower pH, which would normally prevent or reverse the cyclization, the diazo functionality

became unstable. As a result, the cyclization was virtually irreversible rendering some of the N- α -free alkyl esters undesirable as DON prodrugs (FIG. 1A).

EXAMPLE 3

5 Prodrug Strategy – Masking Amino Functionality

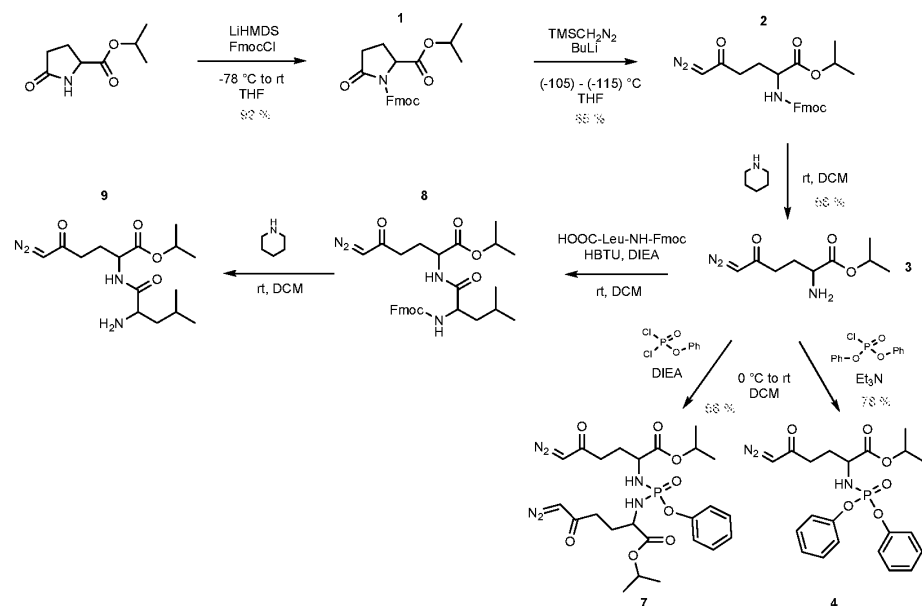
In another embodiment, DON prodrugs were designed by masking only the amino functionalities using N-protected derivatives of Don with an unprotected carboxy functionality. The N-protected derivatives of DON with an unprotected carboxy functionality (FIG. 1B and FIG. 1C) also were not as stable. More
10 particularly, the acidic carboxy functionality caused gradual slow decomposition of the diazo-group. In certain salt forms, the carboxylate anion destabilizes the N- α – protecting group. Even further, as is shown in FIG. 1D, FIG. 1E, FIG. 1F, FIG. 1G and FIG. 1H, many prodrugs tested with a free carboxylate (with the exception of **26**) showed negligible exposure compared to DON when given orally, further suggesting
15 the benefits of derivatizing both carboxylate and amine functionalities for oral availability.

EXAMPLE 4

Synthesis

20 Schemes for synthesis of representative prodrug compounds are shown below (Scheme 1, Scheme 2, Scheme 3, Scheme 4, Scheme 5, Scheme 6, Scheme 7, and Scheme 8).

Scheme 1.



Synthesis of Isopropyl 6-diazo-2-((diphenoxyphosphoryl)amino)-5-oxohexanoate (4)

1-methylethyl 5-oxoprolinate JAM0256. This compound was

5 prepared from known literature. ^1H NMR and ^{13}C NMR spectra were in agreement with the published data.

1-(9H-fluoren-9-ylmethyl) 2-(1-methylethyl) 5-oxopyrrolidine-1,2-dicarboxylate

(1). Referring to scheme 1, the previous compound **JAM0256**

(US2008/107623 A1) (2.94 g, 17.16 mmol) was dissolved in

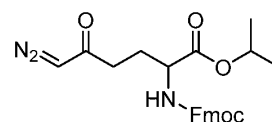
absolute THF (90 mL) under argon and cooled to $-78\text{ }^{\circ}\text{C}$. A solution of LiHMDS (1M in hexanes, 16.3 mL, 16.302 mmol, 0.95 equiv.) was added dropwise and the solution was stirred at the same temperature for 15 min. The resultant yellow mixture was transferred via cannula to a solution of Fmoc chloride (22.2 g, 85.8 mmol, 5 equiv.) in absolute THF (90 mL) at $-78\text{ }^{\circ}\text{C}$. The reaction mixture was stirred at $-78\text{ }^{\circ}\text{C}$ for 2 h. After this period, the reaction was quenched with saturated NH_4Cl (100 mL). Then it was extracted with ethyl acetate (3×50 mL), the combined organic layers were washed with water (40 mL), brine (40 mL) and dried over anhydrous MgSO_4 . The organic solvent was evaporated in vacuo. The residue was chromatographed on silica gel (hexane – ethyl acetate 2 : 1) to afford the desired product **1** (6.2 g, 92 %) as a colorless solid. $^1\text{H NMR}$ (400 MHz, CDCl_3): 1.23 (3H, d, $J = 6.2$), 1.26 (3H, d, $J = 6.3$), 2.06 – 2.13 (1H, m), 2.34 – 2.45 (1H, m).

- 2.53 – 2.61 (1H, m), 2.67 – 7.76 (1H, m), 4.31 (1H, t, $J = 7.5$), 4.40 – 4.44 (1H, m), 4.53 – 4.57 (1H, m), 4.65 (1H, dd, $J = 9.4, 2.6$), 5.07 (1H, hept, $J = 6.3$), 7.31 – 7.35 (2H, m), 7.39 – 7.43 (2H, m), 7.71 – 7.78 (4H, m). ^{13}C NMR (101 MHz, CDCl_3): 21.69, 21.78, 22.01, 31.31, 46.64, 58.99, 69.20, 69.78, 120.06, 120.08, 125.43, 125.57, 127.32 (2C), 127.98 (2C), 141.31, 141.33, 143.39, 143.43, 151.56, 170.58, 172.92. **Optical rotation:** $[\alpha]_{\text{D}}^{22} - 24.1^\circ$ (c 0.332, CHCl_3). **IR** (CHCl_3): 3068 w, 3029 m, 2985 m, 2939 w, 2883 vw, 1797 s, 1758 s, sh, 1739 vs, 1724 vs, sh, 1609 vw, 1580 vw, 1479 w, 1463 m, 1452 s, 1421 w, 1386 s, 1377 m, 1305 vs, 1194 m, 1105 s, 1045 m, 1033 m, 621 w, 425 w cm^{-1} . **ESI MS:** 416 ($[\text{M} + \text{Na}]^+$). **HR ESI MS:** calcd for $\text{C}_{23}\text{H}_{23}\text{O}_5\text{NNa}$ 416.14684; found 416.14694.

Isopropyl 2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-diazo-5-

oxohexanoate (2). Referring to scheme 1, trimethylsilyl

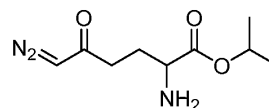
diazomethane solution (2M in diethyl ether, 6 mL, 11.93 mmol, 1.2 equiv.) was dissolved in absolute THF (55



- mL) under argon and cooled to -98°C . A solution of *n*-butyllithium (2.5 M in hexanes, 4.9 mL, 12.23 mmol, 1.23 equiv.) was added dropwise and the solution was stirred at -98°C for 30 min. The resultant mixture was transferred via cannula to a solution of previous compound **1** (3.91 g, 9.94 mmol, 1 equiv.) in absolute THF (100 mL) at -116°C . The reaction mixture was slowly warmed to -78°C and then quenched with saturated NH_4Cl . Then it was extracted with ethyl acetate (3×50 mL), the combined organic layers were washed with water (40 mL), brine (40 mL) and dried over anhydrous MgSO_4 . The organic solvent was evaporated in vacuo. The residue was chromatographed on silica gel (chloroform – acetone 20:1) to afford the desired product **2** (3.68 g, 85 %) as a yellowish solid. ^1H NMR (400 MHz, CDCl_3): 1.25 – 1.28 (6H, m), 1.95 – 2.04 (1H, m), 2.17 – 2.26 (1H, m), 2.31 – 2.52 (2H, m), 4.22 (1H, t, $J = 7.1$), 4.29 – 4.43 (3H, m), 5.06 (1H, hept, $J = 6.1$), 5.27 (1H, s), 5.59 (1H, d, $J = 8.2$), 7.30 – 7.34 (2H, m), 7.38 – 7.42 (2H, m), 7.59– 7.62 (2H, m), 7.75 – 7.77 (2H, m). ^{13}C NMR (101 MHz, CDCl_3): 21.81, 21.84, 27.69, 36.56, 47.21, 53.67, 67.10, 69.62, 120.08, 120.09, 125.18, 125.21, 127.16 (2C), 127.81 (2C), 141.35, 141.37, 143.75, 143.96, 156.16, 171.50, 193.67. **Optical rotation:** $[\alpha]_{\text{D}}^{22} + 15.1^\circ$ (c 0.674, CHCl_3). **IR** (CHCl_3): 3428 m, 3116 w, 3068 w, 2985 m, 2940 w, 2882 w, 2110 vs, 1731 vs, sh, 1719 vs, 1641 s, 1608 w, sh, 1580 vw, 1509 s, 1478 m, 1466 m, 1451 s, 1418 w, sh, 1386 s, sh, 1377 s, 1349 s, 1232 s, 1105 s, 1052 s, 1033 m, 622 w, 539

m, 488 m, 426 w cm^{-1} . **ESI MS**: 458 ($[\text{M} + \text{Na}]^+$). **HR ESI MS**: calcd for $\text{C}_{24}\text{H}_{25}\text{O}_5\text{NaN}$ 458.16864; found 458.16873.

Isopropyl 2-amino-6-diazo-5-oxohexanoate (3). Referring to scheme 1, the previous compound **2** (900 mg, 2.07 mmol), was dissolved in dichloromethane (10 mL). Piperidine (514 μL , 5.17 mmol, 2.5 equiv.) was added and the reaction mixture

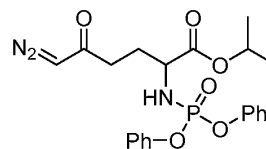


was stirred at room temperature for 4 h. The organic solvent was evaporated in vacuo. The residue was chromatographed on silica gel (chloroform - methanol 30:1) to afford the desired product (290 mg, 66 %) as a yellow oil. **^1H NMR** (400 MHz, CDCl_3): 1.21 (3H, d, $J = 1.8$), 1.23 (3H, d, $J = 1.8$), 1.52 (2H, bs), 1.74 – 1.85 (1H, m), 2.02 – 2.10 (1H, m), 2.36 – 2.53 (2H, bm), 3.37 (1H, dd, $J = 8.4, 5.0$), 5.00 (1H, hept, $J = 6.3$), 5.27 (1H, s). **^{13}C NMR** (101 MHz, CDCl_3): 21.87, 21.89, 29.65, 36.99, 53.94, 68.64, 175.21, 194.25. **Optical rotation**: $[\alpha]_D^{22} + 6.5^\circ$ (c 0.444, CHCl_3). **IR** (CHCl_3): 3390 w, 3323 vw, 3116 w, 2984 s, 2939 m, 2877 w, 2109 vs, 1725 vs, 1640 s, 1467 m, 1454 m, 1439 w, sh, 1388 s, sh, 1376 vs, 1349 s, 1199 s, 1106 vs cm^{-1} . **ESI MS**: 236 ($[\text{M} + \text{Na}]^+$); **HR ESI MS**: calcd for $\text{C}_9\text{H}_{15}\text{O}_3\text{N}_3\text{Na}$ 236.1006; found 236.1007.

Isopropyl 6-diazo-2-((diphenoxyphosphoryl)amino)-5-oxohexanoate (4).

Referring to scheme 1, isopropyl DON **3** (80 mg, 0.38

mmol) was dissolved in absolute dichloromethane (4 mL) and triethylamine (210 μL , 1.5 mmol, 4 equiv.) was added. This solution was cooled to 0 $^\circ\text{C}$ and diphenyl



chlorophosphate (156 μL , 0.75 mmol, 2 equiv.) was added dropwise. The reaction mixture was stirred at 0 $^\circ\text{C}$ for 30 min and then the cooling bath was removed. The reaction mixture was then stirred at room temperature for 2 h. The organic solvent was evaporated *in vacuo*. The residue was chromatographed on silica gel (chloroform - acetone 30:1) to afford the desired product (131 mg, 78 %) as a yellow solid. **^1H NMR** (400 MHz, CDCl_3): 1.19 (3H, d, $J = 1.0$), 1.21 (3H, d, $J = 1.0$), 1.84 – 1.93 (1H, m), 2.07 – 2.16 (1H, m), 2.19 – 2.41 (2H, m), 3.79 – 3.84 (1H, m), 4.97 (1H, hept, $J = 6.2$), 5.07 (1H, s). **^{13}C NMR** (101 MHz, CDCl_3): 21.75, 21.81, 29.34, 35.88, 54.29, 69.80, 80.90, 120.32 (2C, d, $J_{\text{C,P}} = 5.0$), 120.39 (2C, d, $J_{\text{C,P}} = 4.9$), 125.23 (2C, d, $J_{\text{C,P}} = 1.0$), 125.28 (2C, d, $J_{\text{C,P}} = 1.0$), 130.00 (2C), 150.68 (d, $J_{\text{C,P}} = 5.8$), 150.75 (d, $J_{\text{C,P}} = 6.2$), 171.93 (d, $J_{\text{C,P}} = 5.9$), 193.51. **^{31}P NMR** (101 MHz, CDCl_3): 0.32. **Optical rotation**: $[\alpha]_D^{22} + 15.1^\circ$ (c 0.337, CHCl_3). **IR** (CHCl_3): 3383 w, 3115 w, 3101 vw,

3063 vw, 2985 m, 2938 w, 2878 vw, 2110 vs, 1731 s, 1642 s, 1600 m, sh, 1591 m, 1490 s, 1467 w, 1456 m, 1448 vw, sh, 1426 m, 1385 s, sh, 1377 s, 1350 m, 1191 vs, 1163 s, 1071 w, 1026 m, 941 vs, 904 m, 821 w, 690 m, 617 w, 487 m cm⁻¹. **ESI MS**: 468 ([M + Na]⁺). **HR ESI MS**: calcd for C₂₁H₂₄O₆N₃NaP 468.12949; found

5 468.12952.

Synthesis of ethyl 2-((((4-acetoxybenzyl)oxy)carbonyl)amino)-6-diazo-5-oxohexanoate (6)

1-[4-(acetyloxy)benzyl] 2-ethyl 5-oxopyrrolidine-

1,2-dicarboxylate (5). To a solution of phosgene

10 (15% wt in toluene, 10 mL, 14 mmol, 2.5 equiv.)

cooled to 0 °C was added a solution of 4-

Acetoxybenzyl alcohol (934 mg, 5.6 mmol) in

toluene (6.7 mL). The reaction mixture was stirred at the same temperature overnight.

The volatiles were removed *in vacuo* and the product 4-(Acetoxy)benzyl

15 Chloroformate was dissolved in THF (5 mL) and used without any purification. Ethyl

pyrrolglutamate (800 mg, 5.1 mmol) was dissolved in absolute THF (13 mL) under

argon and cooled to -78 °C. A solution of LiHMDS (1M in hexanes, 6.12 mL, 6.12

mmol, 1.2 equiv.) was added dropwise and the solution was stirred at the same

temperature for 15 min. The resultant yellow mixture was transferred via cannula to a

20 solution of 4-(Acetoxy)benzyl chloroformate (5.6 mmol, 1 equiv) at -78 °C. The

reaction mixture was stirred at -78 °C for 2 h. After this period, the reaction was

quenched with saturated NH₄Cl (100 mL). Then it was extracted with ethyl acetate (3

× 50 mL), the combined organic layers were washed with water (40 mL), brine (40

mL) and dried over anhydrous MgSO₄. The organic solvent was evaporated in *vacuo*.

25 The residue was chromatographed on silica gel (hexane – ethyl acetate 1:1) to afford

the desired product **5** (1.16 g, 65 %) as a colorless solid. **¹H NMR** (400 MHz, CDCl₃):

1.16 (3H, t, *J* = 7.2), 1.99 – 2.06 (1H, m), 2.25 (3H, s), 2.28 – 2.36 (1H, m), 2.42 –

2.50 (1H, m), 2.55 – 2.64 (1H, m), 4.11 (1H, qd, *J* = 7.1, 2.6), 4.62 (1H, dd, *J* = 9.4,

2.7), 5.16 (1H, d, *J* = 12.4), 4.25 (1H, d, *J* = 12.4), 7.02 – 7.05 (2H, m), 7.35 – 7.39

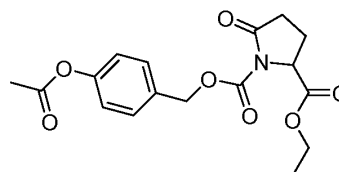
30 (2H, m). **¹³C NMR** (101 MHz, CDCl₃): 14.02, 21.08, 21.79, 30.99, 58.74, 61.82,

67.58, 121.75, 129.46, 132.62, 150.70, 150.83, 169.28, 170.97, 172.93. **Optical**

rotation: [α]_D²² - 30.7° (c 0.298, CHCl₃). **IR** (CHCl₃): 2968 w, 2942 vw, 2876 vw,

1797 s, 1753 vs, 1717 s, sh, 1609 w, 1597 vw, 1510 m, 1476 vw, 1463 w, 1450 w,

1447 w, 1421 w, 1402 w, sh, 1380 m, 1372 m, 1303 s, 1288 s, 1259 m, 1198 vs, 1166

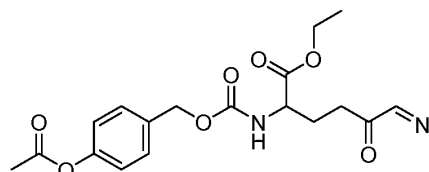


s, 1107 vw, 1045 m, 1019 m, 1012 m, sh, 913 w, 846 w, 596 w, cm^{-1} . **ESI MS**: 372 ($[\text{M} + \text{Na}]^+$). **HR ESI MS**: calcd for $\text{C}_{17}\text{H}_{19}\text{O}_7\text{NNa}$ 372.10537; found 372.10541.

Ethyl 2-(((4-acetoxymethyl)oxy)carbonylamino)-6-diazo-5-oxohexanoate (6).

Trimethylsilyl diazomethane solution (2M

5 in diethyl ether, 1.7 mL, 3.43 mmol, 1.2 equiv.) was dissolved in absolute THF (17 mL) under argon and cooled to $-98\text{ }^{\circ}\text{C}$. A

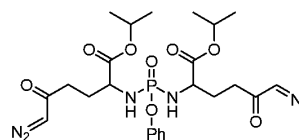


solution of *n*-butyllithium (2.5 M in hexanes, 1.4 mL, 3.52 mmol, 1.23 equiv.) was added dropwise and the solution was stirred at $-98\text{ }^{\circ}\text{C}$ for 30 min. The resultant mixture was transferred via cannula to a solution of previous compound **5** (1.0 g, 2.86 mmol, 1 equiv.) in absolute THF (27 mL) at $-116\text{ }^{\circ}\text{C}$. The reaction mixture was slowly warmed to $-78\text{ }^{\circ}\text{C}$ and then quenched with saturated NH_4Cl . Then it was extracted with ethyl acetate ($3 \times 50\text{ mL}$), the combined organic layers were washed with water (40 mL), brine (40 mL) and dried over anhydrous MgSO_4 . The organic solvent was evaporated in vacuo. The residue was chromatographed on silica gel (chloroform – acetone 20:1) to afford the desired product **6** (460 mg, 41 %) as a yellowish solid. **^1H NMR** (400 MHz, CDCl_3): 1.25 (3H, t, $J = 7.1$), 1.92 – 2.03 (1H, m), 2.13 – 2.23 (1H, m), 2.28 (3H, s), 2.32 – 2.43 (1H, m), 4.18 (1H, q, $J = 7.1$), 4.29 – 4.34 (1H, m), 5.04 (1H, d, $J = 12.4$), 5.10 (1H, d, $J = 12.1$), 5.22 (1H, s), 7.31 (1H, d, $J = 8.2$), 7.04 – 7.07 (2H, m), 7.34 – 7.37 (2H, m). **^{13}C NMR** (101 MHz, CDCl_3): 14.22, 21.19, 27.53, 36.45, 53.62, 61.78, 66.38, 121.78, 129.52, 134.01, 150.59, 156.01, 169.51, 171.90, 193.60. **Optical rotation**: $[\alpha]_D^{22} + 15.5^{\circ}$ (c 0.129, CHCl_3).

IR (CHCl_3): 3428 w, 3116 w, 2966 w, 2110 s, 1721 vs, 1742 s, sh, 1641 m, 1609 w, sh, 1595 vw, sh, 1509 s, 1418 vw, 1381 s, sh, 1371 s, 1344 m, 1197 vs, 1166 m, 1106 vw, 1053 m, br, 1019 m, 1012 m, sh, 913 w, 848 w, 595 w, 492 w cm^{-1} . **ESI MS**: 414 ($[\text{M} + \text{Na}]^+$). **HR ESI MS**: calcd for $\text{C}_{18}\text{H}_{21}\text{O}_7\text{N}_3\text{Na}$ 414.12717; found 414.12713.

Synthesis of 1-methylethyl 2,6-bis[4-(1 λ^5 -diazynylidene)-3-oxobutyl]-9-methyl-7-oxo-4-phenoxy-8-oxa-3,5-diaza-4-phosphadecan-1-oate 4-oxide (7)

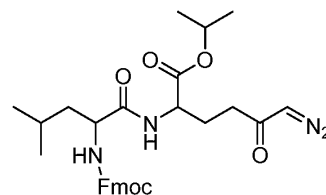
1-methylethyl 2,6-bis[4-(1 λ^5 -diazynylidene)-3-oxobutyl]-9-methyl-7-oxo-4-phenoxy-8-oxa-3,5-diaza-4-phosphadecan-1-oate 4-oxide (7). Referring to scheme 1, isopropyl DON **3** (100 mg, 0.38 mmol) was dissolved in absolute dichloromethane (3 mL) and diisopropylethyl amine (327 μL , 1.88 mmol, 4



equiv.) was added. This solution was cooled to 0 °C and phenyl dichlorophosphate (31.6 μ L, 0.21 mmol, 0.45 equiv.) was added dropwise. The reaction mixture was stirred at 0 °C for 30 min and then the cooling bath was removed. The reaction mixture was then stirred at room temperature for 2 h. The organic solvent was evaporated *in vacuo*. The residue was chromatographed on silica gel (ethyl acetate - methanol 40:1) to afford the desired product (78 mg, 66 %) as a yellow oil. **¹H NMR** (400 MHz, CDCl₃): 1.21 – 1.24 (12H, m), 1.81 – 1.93 (2H, m), 2.09 – 2.19 (2H, m), 2.24 – 2.52 (4H, m), 3.61 – 3.70 (2H, m), 3.90 – 3.98 (2H, m), 4.99 (1H, hept, *J* = 6.3) 4.99 (1H, hept, *J* = 6.2), 5.24 (1H, s), 5.33 (1H, s), 7.10 – 7.14 (1H, m), 7.17 – 7.20 (2H, m), 7.28 – 7.31 (2H, m). **¹³C NMR** (101 MHz, CDCl₃): 21.81 (4C), 29.26, 29.43, 36.21 (2C), 53.78 (d, *J*_{C,P} = 1.7), 53.97, 69.54, 69.66, 120.43 (d, *J*_{C,P} = 4.8), 124.76, 129.76 (2C), 151.03 (2C, d, *J*_{C,P} = 6.8), 172.47 (d, *J*_{C,P} = 5.5), 172.64 (d, *J*_{C,P} = 5.2), 194.07 (2C). **³¹P NMR** (101 MHz, CDCl₃): 11.08. **Optical rotation**: [α]_D²² + 6.4° (c 0.313, CHCl₃). **IR** (CHCl₃): 3099 w, 3303 w, 2104 vs, 1732 s, 1639 s, 1592 w, 1492 m, 1385 sh, m, 1376 s, 1240 sh, m, 1211 s, 1183 sh, m, 1167 m, 1144 m, 1132 sh, m, 1106 s, 1072 w, 1025 w, 1006 m, 923 m, 832 sh, w, 771 w, m, 692 w cm⁻¹. **ESI MS**: 587 ([M + Na]⁺). **HR ESI MS**: calcd for C₂₄H₃₃N₆O₈PNa 587.19897; found 587.19899.

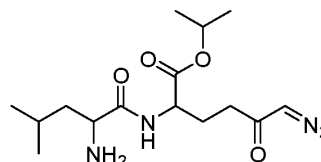
Synthesis of isopropyl 2-(2-(2-amino-4-methylpentanamido)-6-diazo-5-oxohexanoate (9)

Isopropyl 2-(2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-methylpentanamido)-6-diazo-5-oxohexanoate (8). Referring to scheme 1, Fmoc-NH-(*L*)-Leu-COOH (874 mg, 2.47 mmol, 1.1 eq.) and HBTU (1023 mg, 2.70 mmol, 1.2 eq.) were suspended in dry DCM (15 mL). DIEA (872 mg, 1.18 mL, 6.75 mmol, 3 eq.) and then the solution of NH₂-DON-COOiPr (448 mg, 2.25 mmol, 1 eq.) in dry DCM (5 mL) were added by syringe. The reaction mixture was stirred for 2 h at room temperature under an inert atmosphere. DCM (20 mL) was added and the organic phase was washed with sat. NaHCO₃ (40 mL), 1M HCl (40 mL), water (2x40 mL) and sat. NaCl (40 mL), dried over MgSO₄. DCM was evaporated. The crude product was purified by column chromatography (DCM/EtOAc 4:1, *R*_f 0.27) and light yellow solid (949 mg) was obtained in a 79 % yield. **¹H NMR** (400 MHz, CDCl₃): 0.91 – 0.96 (6H, m), 1.23 – 1.25 (6H, m), 1.51 – 1.73 (3H, m), 1.93 – 2.02 (1H, m), 2.14 – 2.24 (1H, m), 2.26 – 2.44 (2H, m), 4.16 – 4.24 (2H, m), 4.33 – 4.37 (1H, m),



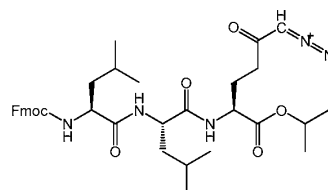
4.39 – 4.44 (1H, m), 4.47 – 4.52 (1H, m), 4.96 – 5.08 (1H, m), 5.18 (1H, s), 5.36 (1H, d, $J = 8.2$), 6.84 (1H, d, $J = 7.8$), 7.30 (2H, tt, $J = 7.4, 1.2$), 7.37 – 7.42 (2H, m), 7.58 (2H, d, $J = 7.4$), 7.75 (2H, d, $J = 7.5$). ^{13}C NMR (101 MHz, CDCl_3): 21.70, 21.72, 22.04, 22.92, 24.66, 27.04, 36.37, 41.75, 47.14, 52.05, 53.56, 67.08, 69.47, 119.98, 120.01, 125.06, 125.13, 127.10, 127.11, 127.74, 127.75, 141.28 (2C), 143.76, 143.81, 156.14, 170.95, 172.29, 193.80. **Optical rotation:** $[\alpha]_{\text{D}}^{22} - 6.1^\circ$ (c 0.472, CHCl_3). **IR** (CHCl_3): 3304 m, sh, 3067 w, 3018 w, 2105 s, 1730 s, 1704 s, 1659 vs, 1639 sh, m, 1612 sh, w, 1580 sh, w, 1539 s, 1478 m, 1451 m, 1467 m, 1386 sh, s, 1375 s, 1244 s, 1172 sh, m, 1145 m, 1106 s, 834 sh, w, 759 m, 740 s, 621 m, 427 cm^{-1} . **ESI MS:** 571 ($[\text{M} + \text{Na}]^+$). **HR ESI MS:** calcd for $\text{C}_{24}\text{H}_{33}\text{O}_4\text{N}_4\text{Na}$ 571.25271; found 571.25271.

Isopropyl 2-(2-amino-4-methylpentanamido)-6-diazo-5-oxohexanoate (9). The same method of preparation as for previous compound **3** was used. Referring to scheme 1, compound **8** (90 mg, 0.164 mmol), dichloromethane (1 mL), piperidine (41 μL 0.41 mmol, 2.5 equiv.). Chromatography on silica gel (chloroform - methanol 20:1). Product (31 mg, 66 %) as a yellow solid. ^1H NMR (400 MHz, CDCl_3): 0.94 (3H, d, $J = 6.3$), 0.98 (3H, d, $J = 6.4$), 1.25 (3H, d, $J = 2.8$), 1.26 (3H, d, $J = 2.8$), 1.35 – 1.42 (1H, m), 1.76 – 1.79 (2H, m), 1.95 – 2.04 (1H, m), 2.17 – 2.25 (1H, m), 2.33 – 2.49 (2H, m), 3.48 (1H, dd, $J = 9.7, 4.1$), 4.52 (1H, td, $J = 8.5, 4.7$), 5.04 (1H, hept, $J = 6.3$), 5.35 (1H, s), 7.87 (1H, d, $J = 8.3$). ^{13}C NMR (101 MHz, CDCl_3): 27.59, 28.19, 33.40, 66.39, 80.90, 124.87, 128.33, 128.35, 128.66, 136.10, 140.58, 166.01, 172.75. **Optical rotation:** $[\alpha]_{\text{D}}^{22} +^\circ$ (c 0.33, CH_2Cl_2). **IR** (CHCl_3): 3412 w, 3343 w, 2110 vs, 1731 s, 1663 s, 1643 sh, s, 1603 sh, w, 1510 s, 1413 w, 1386 sh, s, 1376 sh, s, 1370 sh, s, 1349 m, 1145 m, 1105 s cm^{-1} . **ESI MS:** 327 ($[\text{M} + \text{H}]^+$). **HR ESI MS:** calcd for $\text{C}_{15}\text{H}_{27}\text{O}_4\text{N}_4\text{Na}$ 327.20268; found 327.20280.



Synthesis of 1-methylethyl L-leucyl-L-leucyl-6-(1⁵-diazynylidene)-5-oxo-L-norleucinate (11)

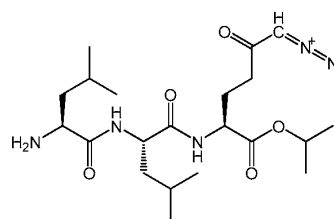
1-Methylethyl N-[(9H-fluoren-9-ylmethoxy)carbonyl]-L-leucyl-L-leucyl-6-(1⁵-diazynylidene)-5-oxo-L-norleucinate (10). Fmoc-NH-(L)-Leu-COOH (224 mg, 0.634 mmol, 1.1 equiv.) and 2-benzotriazol-1-yl-tetramethyluronium tetrafluoroborate (TBTU)



(222 mg, 0.69 mmol, 1.2 equiv.) were suspended in absolute dichloromethane (5 mL) and diisopropylethyl amine (301 μ L, 1.73 mmol, 3 equiv.) was added. The reaction mixture was stirred at room temperature for 30 min. and then the solution of **9** (188 mg, 0.58 mmol) in dry dichloromethane (3 mL) were added by syringe. The reaction mixture was stirred for 2 h at room temperature under inert atmosphere. The organic solvent was evaporated *in vacuo*. The residue was chromatographed on silica gel (hexane:ethyl acetate, 1:1) to afford the desired product **10** (188 mg, 49 %) as a yellowish amorphous solid. **¹H NMR** (400 MHz, CDCl₃): 0.86 – 0.98 (12H, m), 1.24 (3H, d, *J* = 5.0), 1.25 (3H, d, *J* = 5.0), 1.47 – 1.56 (2H, m), 1.57 – 1.72 (4H, m), 1.91 – 2.04 (1H, m), 2.14 – 2.24 (1H, m), 2.26 – 2.48 (2H, m), 4.14 – 4.22 (1H, m), 4.21 (1H, t, *J* = 6.8), 4.36 – 4.49 (4H, m), 5.03 (1H, hept, *J* = 6.3), 5.14 (1H, d, *J* = 7.5), 5.32 (1H, s), 6.41 (1H, d, *J* = 7.7), 6.83 (1H, d, *J* = 6.8), 7.32 (2H, tt, *J* = 7.4, 1.3), 7.41 (2H, tt, *J* = 7.5, 1.5), 7.58 (2H, d, *J* = 7.5), 7.77 (2H, tt, *J* = 7.6, 1.1). **¹³C NMR** (101 MHz, CDCl₃): 21.82, 21.84, 21.96, 22.22, 22.91, 23.14, 24.83 (2C), 27.22, 36.46, 41.29, 41.39, 47.26, 51.94, 52.12, 53.74, 55.00, 67.22, 69.54, 120.12, 120.14, 125.11, 125.15, 127.22 (2C), 127.88, 127.89, 141.42 (2C), 143.77, 143.89, 156.42, 171.03, 171.81, 172.39, 193.97. **Optical rotation**: [α]_D²² -25.2° (c 0.385, CHCl₃). **IR** (CHCl₃): 3426 m, 3332 w, sh, 3116 w, 3068 w, 2961 s, 2873 m, 2110 s, 1726 vs, 1672 vs, 1640 sh, m, 1610 sh, w, 1579 sh, w, 1541 m, 1506 vs, 1479 m, 1468 m, 1387 s, 1371 sh, s, 1377 s, 1349 m, 1286 m, 1234 s, 1171 sh, m, 1146 m, 1105 s, 1046 m, 1023 sh, w, 824 w, 585 w, 488 w, sh, 426 w cm⁻¹. **ESI MS**: 684 ([M + Na]⁺). **HR ESI MS**: calcd for C₃₆H₄₇O₇N₅Na 684.33677; found 684.33607.

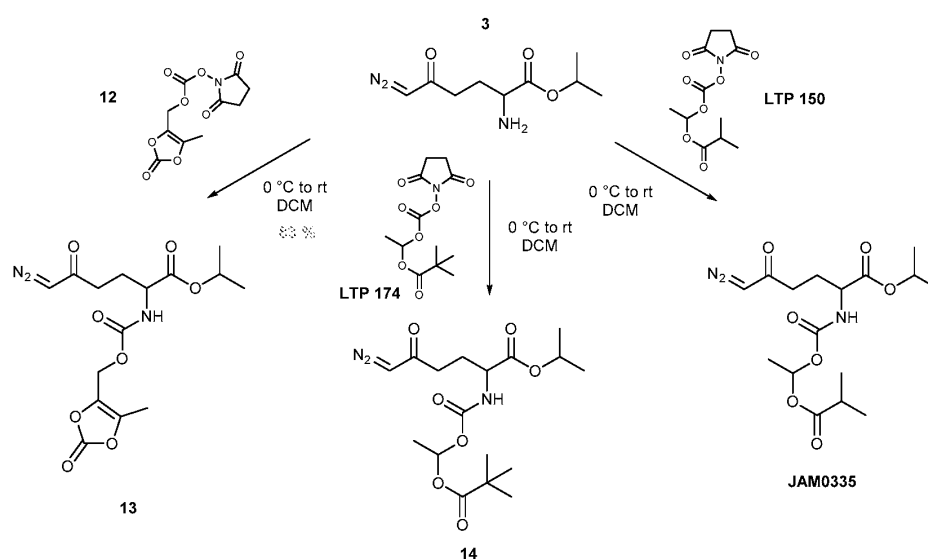
1-Methylethyl L-leucyl-L-leucyl-6-(1⁵-diazynylidene)-5-oxo-L-norleucinate (11).

Compound **10** (180 mg, 0.272 mmol), was dissolved in dichloromethane (4 mL). Piperidine (67 μ L 0.68 mmol, 2.5 equiv.) was added and the reaction mixture was stirred at room temperature for 4 h. The organic solvent was evaporated in *vacuo*. The residue was chromatographed on



silica gel (chloroform:methanol, 30:1) to afford the desired product **11** (80 mg, 67 %) as a yellow amorphous solid. **¹H NMR** (400 MHz, CDCl₃): 0.88 – 0.98 (12H, m), 1.24 (3H, d, *J* = 5.2), 1.26 (3H, d, *J* = 5.3), 1.37 – 1.47 (1H, m), 1.50 – 1.79 (5H, m), 1.91 – 2.07 (1H, m), 2.08 – 2.28 (3H, m), 2.31 – 2.50 (2H, m), 3.52 (1H, dd *J* = 9.5, 3.8), 4.39 (1H, td, *J* = 8.7, 5.3), 4.46 (1H, td, *J* = 8.0, 4.6), 5.03 (1H, hept, *J* = 6.2),

- 5.37 (1H, s), 7.02 (1H, d, $J = 7.4$), 7.77 (1H, d, $J = 8.1$). ^{13}C NMR (101 MHz, CDCl_3): 21.46, 21.83, 21.86, 22.05, 23.09, 23.53, 24.95, 25.01, 27.13, 36.52, 40.89, 43.94, 51.55, 52.16, 53.61, 55.01, 69.49, 171.09, 172.36, 175.91, 194.21. **Optical rotation:** $[\alpha]_D^{22} -31.7^\circ$ (c 0.439, CHCl_3). **IR** (CHCl_3): 3415 w, 3343 w, br, 3117 w, 2984 m, 2961 s, 2936 m, 2873 m, 2855 w, 2110 vs, 1731 s, 1665 vs, br, 1653 vs, br, 1630 s, sh, 1509 s, 1468 m, 1454 w, 1450 w, 1440 w, 1386 s, 1377 s, 1370 s, 1349 m, 1201 m, 1183 w, 1146 m, 1105 s cm^{-1} . **ESI MS:** 440 ($[\text{M} + \text{H}]^+$). **HR ESI MS:** calcd for $\text{C}_{21}\text{H}_{38}\text{O}_5\text{N}_5$ 440.28675; found 440.28674.

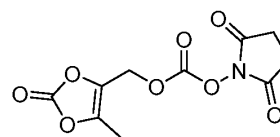
Scheme 2

10

*Synthesis of 1-methylethyl 6-(1 λ^5 -diazynylidene)-N-[(5-methyl-2-oxo-1,3-dioxol-4-yl)methoxy]carbonyl}-5-oxo-L-norleucinate (**13**)*

2,5-Dioxopyrrolidin-1-yl-((5-methyl-2-oxo-1,3-dioxol-4-yl)methyl carbonate (12).

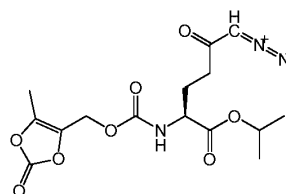
- 15 4-(Hydroxymethyl)-5-methyl-1,3-dioxol-2-one (1.00 g, 7.89 mmol, 1 eq.) was dissolved in dry Et_2O (30 mL) and the solution was cooled to 0°C . At this temperature pyridine (608 mg, 619 μL , 7.69 mmol, 1 eq.) was added and finally the solution of *S*-ethyl carbonochloridothionate (1.04 g, 874 μL , 8.38 mmol, 1.09 eq.) in dry Et_2O (8 mL) was added by dropwise. The mixture was stirred for 1 h at 0°C and overnight at rt (18 h). Et_2O was removed by *vacuo* and DCM (70 mL) was added. The reaction mixture was washed with sat. NaHCO_3 (40 mL) and



- with water (3x40 mL), dried with MgSO₄ and DCM was removed by rotavap. The crude product was purified by column chromatography (hexane/EtOAc 5:1, R_f 0.24). A light yellow liquid (1.29 g) was obtained in 77 % yield. *S*-Ethyl O-((5-methyl-2-oxo-1,3-dioxol-4-yl)methyl) carbonothioate (800 mg, 3.67 mmol, 1 eq.) and *N*-hydroxysuccinimide (844 mg, 7.33 mmol, 2 eq.) were suspended in dry DCM (8 mL). The solution was cooled to 0 °C and peracetic acid (836 mg (100 %), 2.32 g (36 %), 11.00 mmol, 3 eq., 36 % solution in acetic acid) was added by dropwise in 10 minutes. The final mixture was stirred for 30 minutes at 0 °C and 2 h at rt. DCM (20 mL) was added and the organic phase was washed with water (2x35 mL) and sat. NaCl (35 mL). DCM was evaporated and the product **12** was obtained as a colorless solid compound (750 mg) in 76 % yield. ¹H NMR (400 MHz, CDCl₃): 2.20 (3H, s), 2.86 (4H, s), 5.05 (2H, s). ¹³C NMR (101 MHz, CDCl₃): 9.67, 25.58, 59.89, 131.61, 142.20, 151.65, 168.39. IR (CHCl₃): 1842 m, 1824 s, 1819 s, 1792 s, 1749 vs, 1431 w, 1386 w, 1309 m, 1195 s, 935 w, 900 w, 811 vw cm⁻¹. ESI MS: 294 ([M + Na]⁺).
- HR ESI MS: calcd for C₁₀H₉O₈NNa 294.02204; found 294.02213.

1-Methylethyl 6-(1λ⁵-diazynylidene)-*N*-{[(5-methyl-2-oxo-1,3-dioxol-4-yl)methoxy]carbonyl}-5-oxo-L-norleucinate (13**).** Referring to scheme 2, compound

- 12** (70 mg, 0.258 mmol, 1.1 equiv) was dissolved in absolute dichloromethane (2 mL). This solution was cooled to 0 °C and a solution of **3** (50 mg, 0.235 mmol) in dichloromethane (1 mL) was added dropwise.



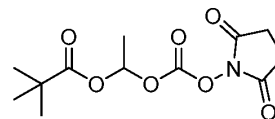
- Reaction mixture was stirred 15 min at 0 °C cooling bath was removed. The reaction mixture was then stirred at room temperature for 1 h. The organic solvent was evaporated *in vacuo*. The residue was chromatographed on silica gel (chloroform:acetone, 10:1) to afford the desired product **13** (72 mg, 83 %) as a yellow oil. ¹H NMR (400 MHz, CDCl₃): 1.24 (3H, d, *J* = 3.4), 1.26 (3H, d, *J* = 5.6), 1.94 – 2.04 (1H, m), 2.13 – 2.24 (1H, m), 2.16 (3H, s), 2.32 – 2.49 (2H, m), 4.23 – 4.28 (1H, m), 4.77 – 4.87 (2H, m), 5.04 (1H, hept, *J* = 6.3), 5.28 (3H, s), 5.66 (1H, d, *J* = 7.9). ¹³C NMR (101 MHz, CDCl₃): 9.51, 21.82, 21.85, 27.32, 36.33, 53.86, 54.57, 69.79, 133.93, 140.07, 152.31, 155.25, 171.12, 193.50. Optical rotation: [α]_D²² +15.1° (c 0.417, CHCl₃). IR (CHCl₃): 3424 w, 3012 w, 2984 w, 2935 w, 2111 s, 1836 sh, s, 1821 vs, 1736 sh, vs, 1725 vs, 1641 m, 1603 sh, w, 1509 s, 1391 sh, m, 1383 s, 1366 sh, m, 1105 s cm⁻¹. ESI MS: 392 ([M + Na]⁺). HR ESI MS: calcd for C₁₅H₁₉O₈N₃Na 392.10644; found 392.10650.

*Synthesis of 1-methylethyl 6-(1⁵-diazynylidene)-N-({1-[(2,2-dimethylpropanoyl)oxy]ethoxy} carbonyl)-5-oxo-L-norleucinate (**14a**) and (**14b**)*

1-(((2,5-Dioxopyrrolidin-1-yl)oxy)carbonyl)oxyethyl pivalate (LTP 174).

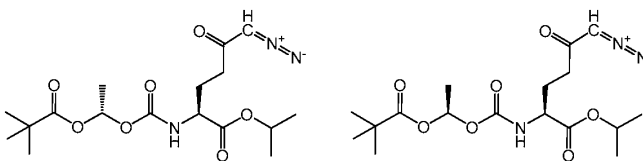
This compound was prepared according to the literature

5 procedure (WO 2008033572 A1).



1-Methylethyl 6-(1⁵-diazynylidene)-N-({1-[(2,2-dimethylpropanoyl)oxy]ethoxy} carbonyl)-5-oxo-L-

10 **norleucinate (**14a**) and (**14b**).** Referring to scheme 2,

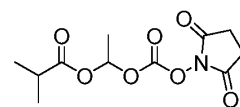


compound **14a** and **14b** were prepared from **3** (200 mg, 0.938 mmol) as described for the preparation of **13** using dichloromethane (8 mL) and 1-(((2,5-dioxopyrrolidin-1-yl)oxy)carbonyl)oxyethyl pivalate (297 mg, 1.032 mmol, 1.1 equiv.), followed by

15 chromatography on silica gel (chloroform:acetone, 20:1) yielding two diastereoisomers **14a** (90 mg, $R_f = 0.25$) and **14b** (50 mg, $R_f = 0.2$) as a yellow oils (39 %). **¹H NMR** (400 MHz, $CDCl_3$): 1.18 (9H, s), 1.25 (3H, d, $J = 3.0$), 1.26 (3H, d, $J = 3.1$), 1.46 (3H, d, $J = 5.4$), 1.94 – 2.04 (1H, m), 2.15 – 2.24 (1H, m), 2.31 – 2.49 (2H, m), 4.24 – 4.30 (1H, m), 5.04 (1H, hept, $J = 6.2$), 5.28 (1H, s), 5.50 (1H, d, $J =$
20 7.9), 6.77 (1H, q, $J = 5.4$). **¹³C NMR** (101 MHz, $CDCl_3$): 19.78, 21.83, 21.85, 27.00 (3C), 27.79, 36.56, 38.79, 53.58, 69.76, 89.70, 153.90, 171.16, 176.61, 193.59. **IR** ($CHCl_3$): 3427 w, 3116 w, 2984 m, 2960 sh, m, 2937 w, 2875 w, 2857 sh, w, 2110 s, 1745 sh, vs, 1730 sh, vs, 1641 m, 1508 s, 1480 m, 1467 sh, w, 1461 w, 1455 sh, w, 1392 sh, s, 1383 sh, s, 1377 s, 1371 sh, s, 1365 sh, s, 1350 m, 1027 m cm^{-1} . **Optical**
25 **rotation (**14a**)**: $[\alpha]^{22}_D +22.0^\circ$ (c 0.191, $CHCl_3$). **Optical rotation (**14b**)**: $[\alpha]^{22}_D +7.6^\circ$ (c 0.158, $CHCl_3$). **ESI MS (**14a**)**: 408 ($[M + Na]^+$). **ESI MS (**14b**)**: 408 ($[M + Na]^+$). **HR ESI MS (**14a**)**: calcd for $C_{17}H_{27}O_7N_3Na$ 408.17412; found 408.17425. **HR ESI MS (**14b**)**: calcd for $C_{17}H_{27}O_7N_3Na$ 408.17412; found 408.17421. Diastereomer **14a** was used for all biological studies.

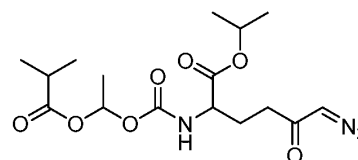
30 *Synthesis of Isopropyl 6-diazo-2-(((1-((3-methylbutanoyl)oxy)ethoxy)carbonyl)amino)-5-oxohexanoate (JAM0335)*
1-(((2,5-Dioxopyrrolidin-1-yl)oxy)carbonyl)oxyethyl isobutyrate (LTP 150)

This compound was prepared according the literature procedure (Magill et al., 1957).



Isopropyl 6-diazo-2-(((1-(3-methylbutanoyl)oxy)ethoxy)carbonyl)amino)-5-oxohexanoate (JAM0335). The same

method of preparation as for previous compound **13** was used. Referring to scheme 2, **LTP150** (74 mg, 0.258 mmol, 1.1 equiv.),

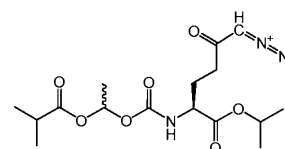


dichloromethane (2 mL). Compound **3** (50 mg, 0.235 mmol), dichloromethane (1 mL), Chromatography on silica gel (chloroform - acetone 20:1). Product (31 mg, 66 %) was isolated as a yellow oil. **¹H NMR** (400 MHz, CDCl₃): 1.48 (9H, s), 2.54 – 2.64 (4H, m), 5.11 (2H, s), 5.48 (1H, m), 6.07 (1H, d, *J* = 1.2), 7.30 – 7.40 (5H, m). **¹³C NMR** (101 MHz, CDCl₃): 27.59, 28.19, 33.40, 66.39, 80.90, 124.87, 128.33, 128.35, 128.66, 136.10, 140.58, 166.01, 172.75. **ESI MS**: 313 ([M + Na]⁺). **HR ESI MS**: calcd for C₁₇H₂₂O₄Na 313.14103; found 313.14103.

*Synthesis of 1-methylethyl 6-(1λ⁵-diazynylidene)-N-({1-[(2-methylpropanoyl)oxy]ethoxy} carbonyl)-5-oxo-L-norleucinate (**15**)*

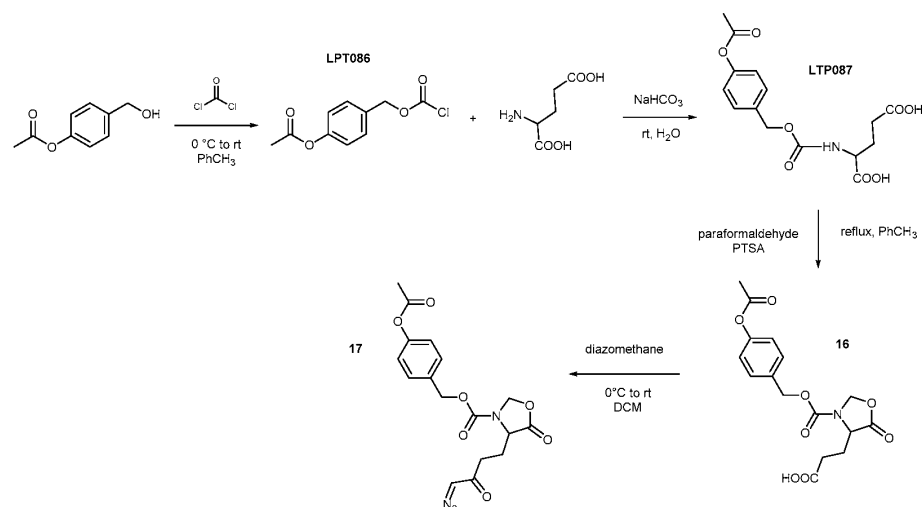
1-Methylethyl 6-(1λ⁵-diazynylidene)-N-({1-[(2-methylpropanoyl)oxy]ethoxy} carbonyl)-5-oxo-L-norleucinate (15**).** Compound **15** was prepared from **3** (300 mg,

1.4 mmol) as described for the preparation of **13** using 1-[[[(2,5-Dioxopyrrolidin-1-yl)oxy]carbonyl]oxy]ethyl 2-methylpropanoate (prepared by WO 2005066122, 423 mg, 1.55 mmol, 1.1 equiv.) and dichloromethane (4 mL),



followed by chromatography on silica gel (chloroform:acetone, 20:1). A yellow oil (426 mg, 82 %). **¹H NMR** (400 MHz, CDCl₃): 1.13 – 1.17 (6H, m), 1.24 – 1.26 (6H, m), 1.46 (3H, d, *J* = 5.4), 1.89 – 2.03 (1H, m), 2.15 – 2.26 (1H, m), 2.31 – 2.58 (3H, m), 4.25 – 4.31 (1H, m), 5.03 (1H, hept, *J* = 6.3), 5.29 and 5.34 (1H, 2 x s), 5.48 and 5.52 (1H, 2x d, *J* = 8.2 and 7.9), 6.75 – 6.81 (1H, m). **¹³C NMR** (101 MHz, CDCl₃): 18.78 (2C), 19.83, 21.81, 21.84, 27.81, 33.98, 36.40, 53.47, 54.92, 69.75, 89.53, 153.87, 171.12, 175.19, 193.60. **IR** (CHCl₃): 3427 w, 3116 w, 2983 m, 2940 w, 2879 w, 2110 s, 1746 vs, 1733 vs, 1641 m, 1509 s, 1449 m, 1387 s, 1349 s, 1341 m, 1321 m, 1030 s cm⁻¹. **ESI MS**: 394 ([M + Na]⁺). **HR ESI MS**: calcd for C₁₆H₂₅O₇N₃Na 394.15847; found 394.15859.

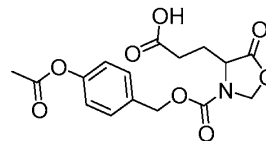
Scheme 3



Synthesis of 4-acetoxybenzyl 4-(4-diazo-3-oxobutyl)-5-oxooxazolidine-3-carboxylate (17)

5 **3-(3-(((4-acetoxybenzyl)oxy)carbonyl)-5-oxooxazolidin-4-yl)propanoic acid (16).**

Referring to scheme 3, a solution of phosgene (15 % vol.) (2.12 g, 2.25 mL, 21.43 mmol, 2.21 eq.) in PhCH₃ (15 mL) was cooled to 0 °C. 4-(Hydroxymethyl)phenyl acetate (1.61 g, 9.70 mmol, 1 eq.) in PhCH₃ (15 mL) was



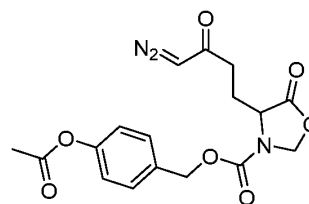
- 10 added dropwise over 15 min. The reaction mixture was stirred for 1 h at 0 °C and then at room temperature overnight (20 h). Solvent was evaporated and the crude product 4-[[[(chlorocarbonyl)oxy]methyl]phenyl acetate (**LTP 086**) was used for the next step without purification. *L*-glutamic acid (951 mg, 6.46 mmol, 1 eq.) was suspended in water (8 mL) and NaHCO₃ (1.37 g, 16.30 mmol, 2.52 eq.) was added in
- 15 few portions. After 15 min of stirring, crude **LTP 086** (2.2 g, 9.70 mmol, 1 eq.) was added by syringe over 2 minutes at room temperature. The mixture was stirred at room temperature overnight (20 h). 1 M HCl was added until the mixture was at a pH of 1 and the water phase was then extracted with EtOAc (10x15 mL). Combined organic fractions were washed with sat. NaCl (150 mL), dried over MgSO₄ and the
- 20 solvent was evaporated. The crude product N-[[[4-(acetoxy)phenyl]methoxy]carbonyl]-*L*-glutamic acid (**LTP 087**) (2.2 g, 6.48 mmol, 1 eq.) was used in the next step without purification. **LTP 087** (2.2 g, 6.48 mmol, 1 eq.) was dissolved in PhCH₃ (45 mL). Paraformaldehyde (389 mg, 12.97

mmol, 2 eq.) and p-Toluenesulfonic acid (PTSA) (123 mg, 0.648 mmol, 0.1 eq.) were added and the mixture was refluxed for 1 h. Toluene was evaporated and the crude product was purified by column chromatography (CHCl₃/MeOH 20:1). The desired product **16** was obtained as a colorless viscous oil (280 mg) in a 12 % yield over three steps.

4-acetoxybenzyl 4-(4-diazo-3-oxobutyl)-5-oxooxazolidine-3-carboxylate (17). The compound **LTP088** (257 mg, 0.732 mmol) was

dissolved in absolute THF (3 mL), cooled to -15 °C and triethylamine (153 µL, 1.097 mmol, 1.5 equiv.)

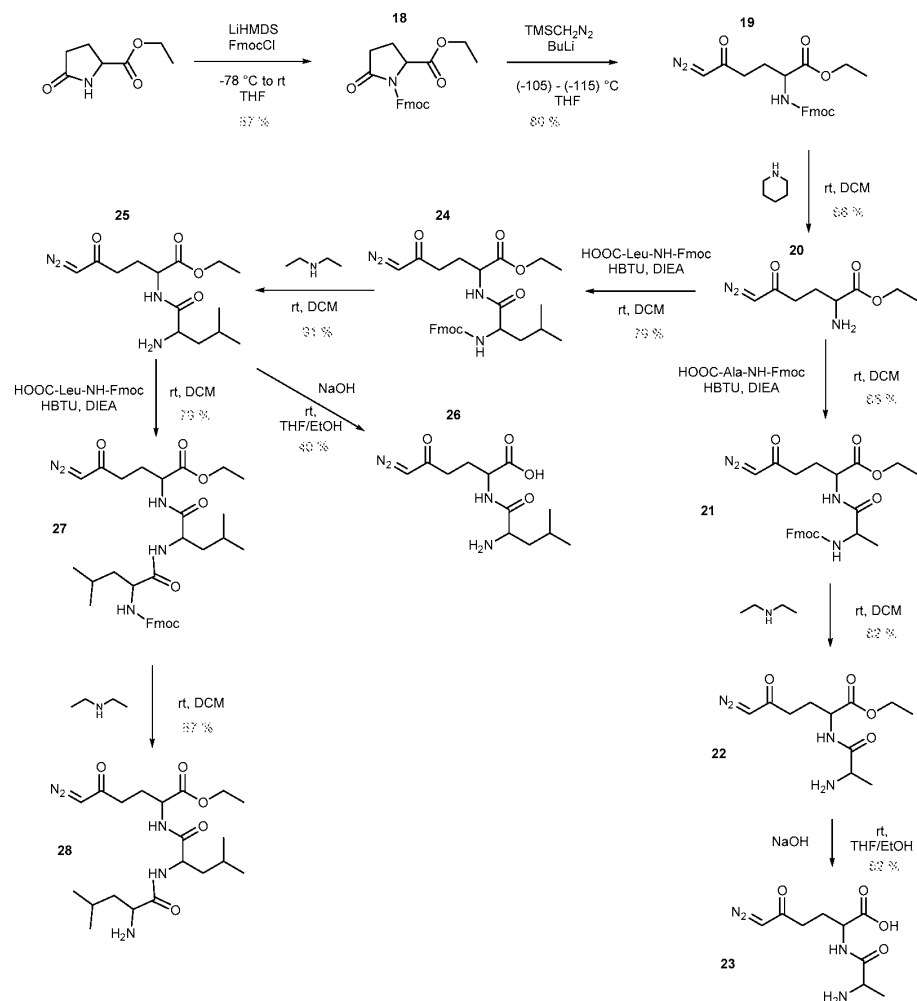
was added dropwise. Then ethylchloroformate was added and the reaction mixture was stirred at -15 °C for 1.5 h. Then a solution of diazomethane was added



and the reaction mixture was stirred for another 30 min at -15 °C and then the cooling bath was removed. The reaction mixture was then stirred at room temperature for 2 h.

The organic solvent was evaporated *in vacuo*. The residue was chromatographed on silica gel (hexane - ethyl acetate 1:1) to afford the desired product (220 mg, 66 %) as a yellow amorphous solid. ¹H NMR (400 MHz, CDCl₃): 2.14 (2H, m), 2.29 (3H, s), 2.32 – 2.56 (2H, m), 4.34 (1H, t, *J* = 6.2), 5.11 – 5.25 (4H, m), 5.51 (1H, s), 7.07 – 7.10 (2H, m), 7.37 – 7.40 (2H, m). ¹³C NMR (101 MHz, CDCl₃): 21.19, 25.80, 35.36, 54.12, 67.49, 77.89, 122.06 (2C), 129.96 (2C), 130.02, 150.97, 152.98, 169.49, 171.81, 192.64. IR (CHCl₃): 3116 w, 2964 vw, 2922 w, 2111 s, 1802 s, 1768 m, sh, 1756 s, 1716 vs, 1642 m, 1610 w, 1597 vw, sh, 1510 m, 1423 m, 1410 s, 1383 m, 1371 s, 1355 s, 1197 vs, 1167 s, 1128 m, 1106 w, 1019 m, 1013 m, 914 m, 850 w, 596 w, 492 w, cm⁻¹. ESI MS: 398 ([M + Na]⁺). HR ESI MS: calcd for C₁₇H₁₇O₇N₃Na 398.09587; found 398.09596.

Scheme 4

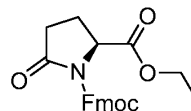


Synthesis of ethyl 2-(2-aminopropanamido)-6-diazo-5-oxohexanoate (22)

1-(9*H*-fluoren-9-ylmethyl)-2-ethyl-5-oxopyrrolidine-1,2-dicarboxylate (18).

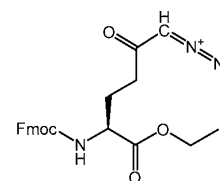
5 Referring to scheme 4, 5-Oxo-L-proline ethyl ester (4.00 g, 25.45 mmol, 1 eq.) was dissolved in absolute THF (120 mL) under inert and cooled to -78 °C. A solution of LiHMDS (1M in THF, 24.2 mL, 24.18 mmol, 0.95 eq.) was added dropwise and the solution was stirred at the same temperature for 20 min. The resultant yellow mixture was transferred via cannula to a solution of Fmoc chloride (32.9 g, 127.3 mmol, 5 eq.) in absolute THF (90 mL) at -78 °C. The reaction mixture was stirred at -78 °C for 2 h and at rt overnight (18 h). After this period the reaction was quenched with saturated NH₄Cl (34 mL) and water (18 mL). Water phase was extracted with EtOAc (60 mL)

10



and combined organic parts were washed with brine (2×100 mL) and dried over anhydrous MgSO₄. The organic solvent was evaporated under vacuo. The residue was chromatographed on silica gel (hexane:EtOAc 3:1 to 1:1) and finally on reverse LC (MeOH:H₂O, 2:1 to 100 % MeOH) to afford the desired product **18** (8.40 g, 87 %) as a colourless solid. ¹H NMR (400 MHz, CDCl₃): 1.26 (3H, d, *J* = 7.1), 2.12 (1H, ddt, *J* = 13.4, 9.4, 2.9), 2.40 (1H, ddt, *J* = 13.4, 10.8, 9.3), 2.57 (1H, ddd, *J* = 17.5, 9.2, 3.1), 2.72 (1H, ddd, *J* = 17.5, 10.7, 9.4), 4.20 (2H, q, *J* = 7.1), 4.30 (1H, t, *J* = 7.3), 4.44 (1H, dd, *J* = 10.5, 7.4), 4.57 (1H, dd, *J* = 10.6, 7.3), 4.65 (1H, dd, *J* = 9.4, 2.5), 7.33 (2H, tt, *J* = 7.4, 1.1), 7.41 (2H, tdd, *J* = 6.9, 1.3, 0.6), 7.71 (1H, dd, *J* = 7.5, 1.0), 7.75 (1H, dd, *J* = 7.5, 1.0), 7.77 (2H, dd, *J* = 7.8, 1.0). ¹³C NMR (101 MHz, CDCl₃): 14.14, 21.96, 31.21, 46.62, 58.76, 61.92, 69.03, 119.99, 120.01, 125.30, 125.44, 127.26 (2C), 127.91 (2C), 141.27, 141.29, 143.35, 143.40, 151.44, 170.80, 172.99. **Optical rotation**: [α]_D²² - 17.2° (c 0.285, CHCl₃). **IR** (CHCl₃): 3068 m, 2985 m, 2941 w, 2898 w, 2875 w, 1797 vs, 1745 vs, br, 1723 vs, 1609 w, 1580 vw, 1478 m, 1463 m, 1452 s, 1400 m, sh, 1385 s, 1197 vs, 1116 vw, sh, 1104 m, 1097 w, sh, 1033 s, 621 m, 426 w, cm⁻¹. **ESI MS**: 402 ([M + Na]⁺). **HR ESI MS**: calcd for C₂₂H₂₁O₅NNa 402.1312; found 402.1313.

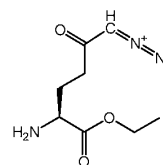
Ethyl 2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-diazo-5-oxohexanoate (19). Trimethylsilyl diazomethane solution (2M in diethyl ether, 7.9 mL, 15.81 mmol, 1.2 eq.) was dissolved in absolute THF (70 mL) under argon and cooled to -98 °C. A solution of *n*-butyllithium (2.5 M in hexanes, 6.5 mL, 16.21 mmol, 1.23 eq.) was added dropwise and the solution was stirred at -98 °C for 30 min. The resultant mixture was transferred via cannula to a solution of **18** (5.00 g, 13.18 mmol, 1 eq.) in absolute THF (120 mL) at -116 °C. The reaction mixture was stirred for 30 min at this temperature and then slowly warmed to -78 °C and quenched with saturated NH₄Cl (150 mL). Water phase was extracted with ethyl acetate (3×50 mL), the combined organic layers were washed with brine (200 mL) and dried over anhydrous MgSO₄. The organic solvent was evaporated in vacuo. The residue was chromatographed on silica gel (CHCl₃:acetone, 20:) to afford the desired product **19** (4.42 g, 80 %) as a yellowish solid. ¹H NMR (400 MHz, CDCl₃): 1.29 (3H, t, *J* = 7.1), 1.93 – 2.10 (1H, m), 2.17 – 2.29 (1H, m), 2.33 – 2.53 (2H, m), 4.22 (3H, t, *J* = 7.1), 4.32 – 4.43 (3H, m), 5.27 (1H, bs), 5.56 (1H, d, *J* = 8.1), 7.32 (2H, tt, *J* = 7.4, 1.3), 7.41 (2H, t, *J* = 7.5), 7.60 (2H, t, *J* = 6.6), 7.77 (2H, dd, *J* = 7.6, 1.0). ¹³C NMR



(101 MHz, CDCl₃): 14.30, 27.63, 36.56, 47.26, 53.63, 54.96, 61.89, 67.17, 120.11, 120.14, 125.22, 125.24, 127.20 (2C), 127.86 (2C), 141.40, 141.43, 143.78, 143.99, 156.18, 172.03, 193.65. **Optical rotation:** $[\alpha]^{22}_{\text{D}} + 10.6^\circ$ (c 0.265, CHCl₃). **IR** (CHCl₃): 3428 w, 3116 w, 3068 w, 2985 w, 2942 w, 2907 vw, 2110 s, 1740 s, sh, 1721 vs, 1642 m, 1510 s, 1478 w, 1465 w, 1451 m, 1381 s, 1105 w, 1052 m, 1033 m, 622 w, 426 w cm⁻¹. **ESI MS:** 444 ([M + Na]⁺). **HR ESI MS:** calcd for C₂₃H₂₃O₅N₃Na 444.15299; found 444.15292.

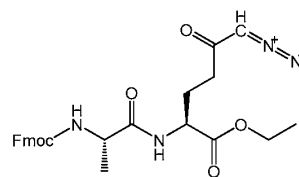
Ethyl 2-amino-6-diazo-5-oxohexanoate (20). Compound **20** was prepared from **19** (100 mg, 0.237 mmol) as described for the preparation of **3** using

dichloromethane (10 mL) and piperidine (58 μ L 0.59 mmol, 2.5 equiv), followed by chromatography on silica gel (chloroform:methanol, 30 : 1). A yellow oil (31 mg, 66 %). **¹H NMR** (400 MHz, CDCl₃): 1.27 (3H, t, $J = 7.1$), 1.55 (2H, bs), 1.78 – 1.88 (1H, m), 2.06 – 2.17 (1H, m), 2.40 – 2.54 (2H, bm), 3.44 (1H, dd, $J = 8.3, 5.1$), 4.17 (2H, q, $J = 7.1$), 5.27 (1H, s). **¹³C NMR** (101 MHz, CDCl₃): 14.24, 29.56, 36.86, 53.77, 54.56, 61.05, 175.58, 194.15. **Optical rotation:** $[\alpha]^{22}_{\text{D}} +^\circ$ (c 0.33, CH₂Cl₂). **IR** (CHCl₃): 3410 w, vbr, 3327, vw, vbr, 2986 m, 2941 w, 2910 w, 2874 w, 2110 s, 1739 vs, 1641 m, 1605 m, sh, 1586 m, 1552 w, 1513 w, br, 1476 w, 1463 m, 1446 m, 1395 m, sh, 1377 s, 1200 s, 1115 m, 1096 m cm⁻¹. **ESI MS:** 182 ([M – H₂O + H]⁺). **HR ESI MS:** calcd for C₁₂H₁₂N₃O₂ 182.0930; found 182.0931.



Ethyl 2-(2-(((9H-Fluoren-9-yl)methoxy)carbonyl)amino)propanamido)-6-diazo-5-oxohexanoate (21). Fmoc-NH-(L)-Ala-COOH (483 mg, 1.55 mmol, 1.1 eq.) and HBTU (642 mg, 1.69 mmol, 1.2 eq.) were suspended in

dry DCM (10 mL). DIEA (547 mg, 737 μ L, 4.32 mmol, 3 eq.) and then the solution of **20** (281 mg, 1.41 mmol, 1 eq.) in dry DCM (3mL) were added by syringe. The reaction mixture was stirred for 2 h at rt under inert atmosphere. DCM (15 mL) was added and the organic phase was washed with sat. NaHCO₃ (25 mL), 1M HCl (25 mL), water (2x25 mL) and sat. NaCl (25 mL), dried over MgSO₄. DCM was evaporated. The crude product was purified by column chromatography (DCM : EtOAc, 1 : 1, R_f 0.40) to give **21** as a light yellow solid (452 mg, 65 %). **¹H NMR** (400 MHz, CDCl₃): 1.28 (3H, t, $J = 7.5$), 1.42 (3H, d, $J = 7.1$), 1.98 – 2.08 (1H, m), 2.16 – 2.28 (1H, m), 2.30 – 2.48 (2H, m), 4.15-4.30 (4H, m),

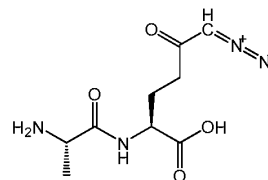
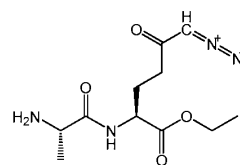


- 4.39 (2H, d, $J = 7.3$), 4.53 (1H, td, $J = 8.0, 4.5$), 5.21 (1H, s), 5.38 (1H, d, $J = 7.4$), 6.81 (1H, d, $J = 7.8$), 7.32 (2H, t, $J = 7.5$), 7.41 (2H, t, $J = 7.5$), 7.60 (2H, d, $J = 7.5$), 7.77 (2H, d, $J = 7.5$). **^{13}C NMR** (101 MHz, CDCl_3): 14.25, 18.86, 27.00, 36.48, 38.74, 47.22, 50.65, 55.11, 61.84, 67.24, 120.11, 120.13, 125.20, 125.24, 127.22 (2C), 127.87 (2C), 141.40 (2C), 143.92 (2C), 156.01, 171.58, 172.47, 193.99. **Optical rotation:** $[\alpha]^{22}_{\text{D}} + 0.4^\circ$ (c 0.225, CHCl_3). **IR** (CHCl_3): 3424 m, 3330 w, br, 3116 vw, 3068 vw, 2986 m, 2941 w, 2908 w, 2875 vw, 2110 s, 1731 vs, 1720 vs, sh, 1682 vs, 1639 m, 1585 vw, 1503 vs, 1478 m, 1451 s, 1377 s, 1233 s, 1116 m, 1105 m, sh, 1095 w, 1032 m, 622 w, 424 w, cm^{-1} . **ESI MS:** 515 ($[\text{M} + \text{Na}]^+$). **HR ESI MS:** calcd for $\text{C}_{26}\text{H}_{28}\text{O}_6\text{N}_4\text{Na}$ 515.19011; found 515.19044.

- Ethyl 2-(2-aminopropanamido)-6-diazo-5-oxohexanoate (22).** Compound **21** (225 mg, 0.457 mmol, 1 eq.) was dissolved in dry DCM (4 mL). Diethylamine (167 mg, 236 μL , 2.28 mmol, 5 eq.) was added by syringe. The reaction mixture was stirred for 6 h at rt under inert atmosphere. DCM was evaporated. The crude product was purified by column chromatography (CHCl_3 :MeOH, 15:1, R_f 0.07) to give **22** as a light yellow solid (101 mg, 82 %). **^1H NMR** (400 MHz, CDCl_3): 1.22 (3H, t, $J = 7.1$), 1.28 (3H, d, $J = 7.0$), 1.89 – 1.94 (2H, m), 1.94 – 2.01 (1H, m), 2.10 – 2.21 (1H, m), 2.27 – 2.43 (2H, m), 3.47 (1H, q, $J = 7.0$), 4.13 (2H, q, $J = 7.1$), 4.47 (1H, td, $J = 8.4, 4.9$), 5.32 (1H, s), 7.81 (1H, d, $J = 8.4$). **^{13}C NMR** (101 MHz, CDCl_3): 14.28, 18.56, 27.44, 36.80, 51.63, 55.05, 58.55, 61.74, 171.90, 175.25, 193.93. **Optical rotation:** $[\alpha]^{22}_{\text{D}} - 31.1^\circ$ (c 0.260, $\text{CHCl}_3 + \text{DMF}$). **IR** (CHCl_3): 3393 w, 3336 vw, br, 3211 vw, br, 3116 vw, 2958 m, 2927 s, 2871 m, 2856 m, 2110 s, 1736 m, 1684 vs, 1639 m, 1517 w, 1379 s, 1115 vw, sh, 1097 w, cm^{-1} . **ESI MS:** 271 ($[\text{M} + \text{H}]^+$). **HR ESI MS:** calcd for $\text{C}_{11}\text{H}_{19}\text{O}_4\text{N}_4$ 271.14008; found 271.14024.

Synthesis of 2-(2-aminopropanamido)-6-diazo-5-oxohexanoic acid (23)

- 2-(2-Aminopropanamido)-6-diazo-5-oxohexanoic acid (23).** Referring to scheme 4, compound **22** (225 mg, 0.457 mmol, 1 eq.) was suspended in EtOH (3 mL) and THF (3 mL). 1M solution of NaOH (16 mg, 397 μL , 0.95 eq.) was added and the mixture was stirred for 15 minutes. The reaction was quenched with 1 M formic acid (397 μL , 0.95 eq.) and after 10 minutes of stirring the mixture was evaporated to dryness. The crude product was purified by preparative HPLC with $\text{Et}_3\text{N}/\text{CH}_3\text{COOH}$ buffer. The desired product **23** was obtained as a light orange solid



(50 mg) in 52 % yield. ¹H NMR (400 MHz, CDCl₃): 1.45 (3H, d, *J* = 7.0), 2.06 – 2.22 (2H, m), 2.45 – 2.56 (2H, m), 4.01 – 4.13 (2H, m), 5.86 (1H, brs).

Synthesis of ethyl 2-((2-amino-4-methylpentanamido)-6-diazo-5-oxohexanoate (25)

Ethyl 2-((2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-methylpentanamido)-6-diazo-5-oxohexanoate (24). Referring to scheme 4, Fmoc-NH-(L)-Leu-COOH

(874 mg, 2.47 mmol, 1.1 eq.) and HBTU (1023 mg, 2.70

mmol, 1.2 eq.) were suspended in dry DCM (15 mL). DIEA (872 mg, 1.18 mL, 6.75 mmol, 3 eq.) and then the

solution of **20** (448 mg, 2.25 mmol, 1 eq.) in dry DCM (5

10 mL) were added by syringe. The reaction mixture was

stirred for 2 h at rt under inert atmosphere. DCM (20 mL) was added and the organic phase was washed with sat. NaHCO₃ (40 mL), 1M HCl (40 mL), water (2x40 mL)

and sat. NaCl (40 mL), dried over MgSO₄. DCM was evaporated. The crude product was purified by column chromatography (DCM : EtOAc, 4 : 1, R_f 0.27) to give **24** as

15 a light yellow solid (949 mg, 79 %). ¹H NMR (400 MHz, CDCl₃): 0.95 (6H, d, *J* = 5.9), 1.26 (3H, t, *J* = 7.5), 1.50 – 1.59 (1H, m), 1.62 – 1.75 (2H, m), 1.95 – 2.05 (1H, m), 2.17 – 2.27 (1H, m), 2.29 – 2.45 (2H, m), 4.14 – 4.25 (4H, m), 4.34 – 4.45 (2H, m), 4.49 – 4.58 (1H, m), 5.19 (1H, s), 5.27 (1H, d, *J* = 8.4), 6.80 (1H, d, *J* = 7.6), 7.31 (2H, t, *J* = 7.4), 7.40 (2H, t, *J* = 7.8), 7.59 (2H, d, *J* = 7.5), 7.76 (2H, d, *J* = 7.6). ¹³C

20 NMR (101 MHz, CDCl₃): 14.12, 22.03, 22.92, 24.66, 26.96, 36.33, 41.65, 47.14, 51.94, 53.56, 55.00, 61.73, 67.09, 120.00, 120.03, 125.05, 125.13, 127.12 (2C), 127.76, 127.77, 141.29, 143.64, 143.75, 143.79, 156.15, 171.46, 172.26, 193.81.

Optical rotation: [α]_D²² - 9.7° (c 0.109, DMF). **IR** (CHCl₃): 3304 m, sh, 3067 w,

3018 w, 2105s, 1730 s, 1704 s, 1659 vs, 1639 sh, m, 1612 sh, w, 1580 sh, w, 1539 s,

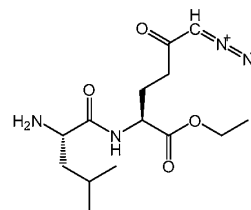
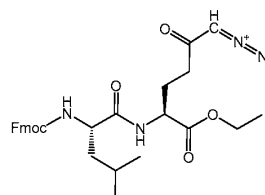
25 1478 m, 1451 m, 1467 m, 1386 sh, s, 1375 s, 1244 s, 1172 sh, m, 1145 m, 1106 s, 834 sh, w, 759 m, 740 s, 621 m, 427 cm⁻¹. **ESI MS:** 557 ([M + Na]⁺). **HR ESI MS:** calcd for C₂₉H₃₄O₆N₄Na 557.23706; found 557.23707.

Ethyl 2-(2-amino-4-methylpentanamido)-6-diazo-5-oxohexanoate (25). Referring to scheme 4, compound **24** (945 mg, 1.77 mmol, 1 eq.) was

30 dissolved in dry DCM (9 mL). Diethylamine (646 mg, 914 μL, 8.84 mmol, 5 eq.) was added by syringe. The reaction mixture was stirred for 4 h at rt under inert atmosphere.

DCM was evaporated. The crude product was purified by

column chromatography (CHCl₃ : MeOH, 15 : 1, R_f 0.38) to give **25** as a colorless



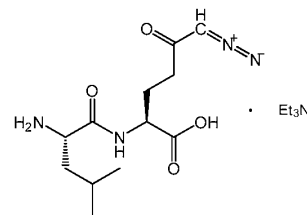
solid (500 mg, 91%). ¹H NMR (400 MHz, CDCl₃): 0.94 (6H, dd, *J* = 14.0, 6.3), 1.27 (3H, t, *J* = 7.1), 1.28 – 1.37 (1H, m), 1.45 (2H, s), 1.57 – 1.83 (2H, m), 1.92 – 2.08 (1H, m), 2.16 – 2.26 (1H, m), 2.26 – 2.49 (2H, m), 3.39 (1H, dd, *J* = 10.0, 3.9), 4.19 (2H, dq, *J* = 7.1, 1.4), 4.54 (1H, dt, *J* = 8.5, 4.8), 5.30 (1H, s), 7.85 (1H, d, *J* = 8.3).

- 5 ¹³C NMR (101 MHz, CDCl₃): 14.25, 21.40, 23.54, 24.97, 27.71, 36.82, 44.29, 51.48, 53.60, 54.86, 61.65, 171.99, 175.99, 193.67. **Optical rotation:** [α]_D²² - 54.8° (c 0.323, CHCl₃). **IR** (CHCl₃): 3412 w, 3343 w, 2110 vs, 1731 s, 1663 s, 1643 sh, s, 1603 sh, w, 1510 s, 1413 w, 1386 sh, s, 1376 sh, s, 1370 sh, s, 1349 m, 1145 m, 1105 s cm⁻¹. **ESI MS:** 335 ([M + Na]⁺). **HR ESI MS:** calcd for C₁₄H₂₅O₄N₄ 313.18703; found
10 313.18712.

Synthesis of 2-(2-amino-4-methylpentanamido)-6-diazo-5-oxohexanoic acid triethylammonium salt (26)

2-(2-Amino-4-methylpentanamido)-6-diazo-5-oxohexanoic acid

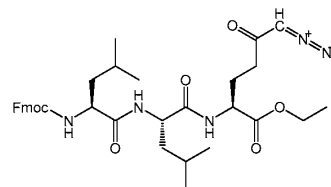
- triethylammonium salt (26).** Compound **25** (134 mg, 0.429 mmol, 1 eq.) was
15 suspended in EtOH (3 mL) and THF (3 mL). 1M solution of NaOH (16 mg, 408 μ L, 0.95 eq.) was added and the mixture was stirred for 15 minutes. The reaction was quenched with 1 M formic acid (408 μ L, 0.95 eq.) and after 10 minutes of stirring the mixture was



- 20 evaporated to dryness. The crude product was purified by preparative HPLC with Et₃N/CH₃COOH buffer. The desired product **26** was obtained as a light orange solid (46 mg) in 40 % yield. ¹H NMR (400 MHz, CDCl₃): 1.10 (6H, d, *J* = 6.4), 1.50 – 1.61 (1H, m), 1.63 – 1.83 (2H, m), 1.96 – 2.06 (1H, m), 2.11 – 2.23 (1H, m), 2.33 – 2.48 (1H, m), 3.67 (1H, dd, *J* = 8.6, 5.6), 4.27 (1H, dd, *J* = 7.0, 5.1), 5.51 (1H, brs).
25 *Synthesis of ethyl 2-(2-(2-amino-4-methylpentanamido)-4-methylpentanamido)-6-diazo-5-oxohexanoate (28).*

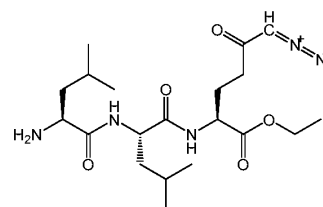
Ethyl 11-(4-diazo-3-oxobutyl)-1-(9H-fluoren-9-yl)-5,8-diisobutyl-3,6,9-trioxo-2-oxa-4,7,10-triazadodecan-12-oate (27). Referring to scheme 4, Fmoc-NH-(L)-Leu-COOH (125 mg, 0.352 mmol, 1.1 eq.) and HBTU

- 30 (146 mg, 0.384 mmol, 1.2 eq.) were suspended in dry DCM (3 mL). DIEA (124 mg, 167 μ L, 0.960 mmol, 3 eq.) and then the solution of **25** (100 mg, 0.320 mmol, 1 eq.) in dry DCM (3 mL) were added by syringe. The reaction mixture was stirred for 3 h at rt under inert atmosphere. DCM



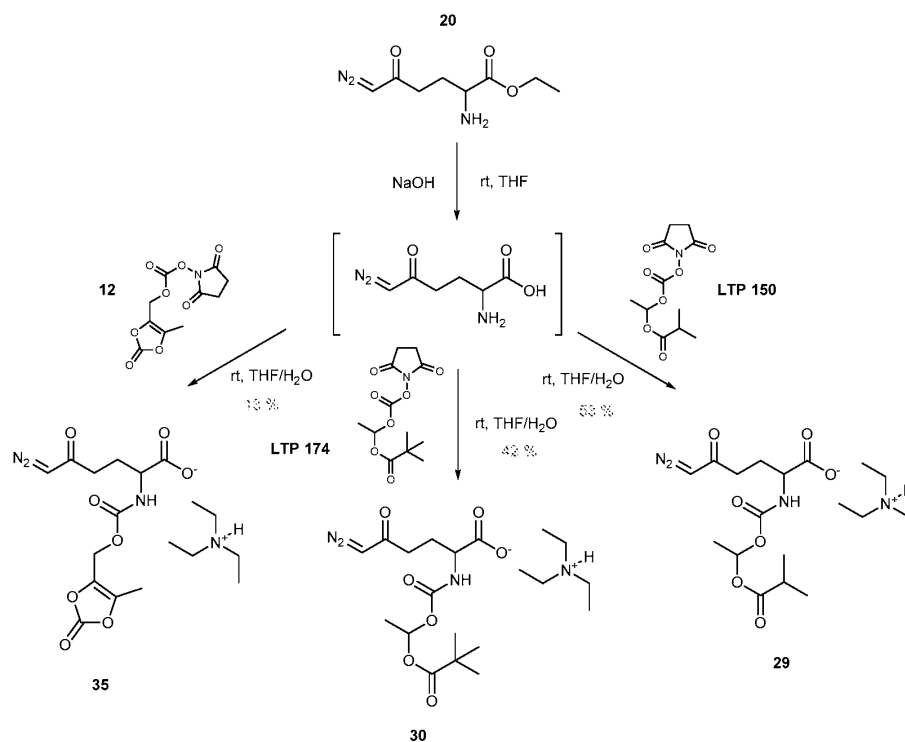
(10 mL) was added and the organic phase was washed with sat. NaHCO_3 (20 mL), 1M HCl (20 mL), water (2x20 mL) and sat. NaCl (20 mL), dried over MgSO_4 . DCM was evaporated. The crude product was purified by column chromatography (DCM : EtOAc, 2 : 1, R_f 0.35) to give **27** as a colorless solid (145 mg, 79 %). **^1H NMR** (400 MHz, CDCl_3): 0.92 (6H, d, $J = 6.5$), 0.95 (6H, d, $J = 6.1$), 1.28 (3H, t, $J = 7.1$), 1.49 – 1.59 (2H, m), 1.60 – 1.74 (4H, m), 1.95 – 2.06 (1H, m), 2.18 – 2.28 (1H, m), 2.30 – 2.50 (2H, m), 4.17 – 4.27 (4H, m), 4.32 – 4.64 (4H, m), 5.29 (1H, d, $J = 8.0$), 5.35 (1H, s), 6.54 (1H, d, $J = 8.0$), 7.00 (1H, d, $J = 7.7$), 7.31 (2H, t, $J = 7.5$, 2.6), 7.40 (2H, td, $J = 7.3$, 2.2), 7.57 (2H, d, $J = 7.5$), 7.76 (2H, d, $J = 7.5$). **^{13}C NMR** (101 MHz, CDCl_3): 14.26, 21.96, 22.17, 22.94, 23.15, 24.85, 27.12, 29.84, 36.48, 38.28, 41.17, 41.33, 47.27, 51.94, 52.03, 61.79, 67.22, 120.14, 120.16, 125.10, 125.14, 127.23 (2C), 127.90, 127.91, 141.43, 143.75, 143.89, 156.43, 171.54, 171.81, 172.39, 194.00. **Optical rotation**: $[\alpha]_D^{22}$ - 34.5° (c 0.109, DMF). **IR** (CHCl_3): 3426 m, 3317 w, br, 3116 w, 3068 w, 2961 s, 2873 m, 2109 s, 1795 w, 1731 vs, 1719 vs, 1667 vs, br, 1635 s, sh, 1508 vs, 1478 m, 1468 m, 1451 s, 1385 s, 1375 s, 1371 s, 1233 s, 1045 m, 1033 m, 622 w, 426 w, cm^{-1} . **ESI MS**: 670 ($[\text{M} + \text{Na}]^+$). **HR ESI MS**: calcd for $\text{C}_{35}\text{H}_{45}\text{O}_7\text{N}_5\text{Na}$ 670.32112; found 670.32122.

Ethyl 2-(2-(2-amino-4-methylpentanamido)-4-methylpentanamido)-6-diazo-5-oxohexanoate (28). Compound **27** (136 mg, 0.210 mmol, 1 eq.) was dissolved in dry DCM (3 mL). Diethylamine (77 mg, 109 μL , 1.05 mmol, 5 eq.) was added by syringe. The reaction mixture was stirred for 8 h at rt under inert atmosphere. DCM was evaporated. The crude product was purified by column chromatography (CHCl₃/MeOH 20:1, R_f 0.30) to give **28** as a light yellow oil (77 mg, 87 %). **^1H NMR** (400 MHz, CDCl_3): 0.92 (6H, t, $J = 6.0$), 0.95 (6H, t, $J = 5.9$), 1.26 (3H, t, $J = 7.1$), 1.34 – 1.41 (1H, m), 1.52 – 1.61 (1H, m), 1.60 – 1.79 (4H, m), 1.95 – 2.03 (1H, m), 2.15 – 2.25 (1H, m), 2.30 – 2.52 (2H, m), 3.48 (1H, dd, $J = 9.5$, 4.1), 3.96 (2H, s), 4.18 (2H, q, $J = 7.1$), 4.41 (1H, td, $J = 8.8$, 5.4), 4.49 (1H, td, $J = 8.0$, 4.6), 5.36 (1H, s), 7.08 – 7.17 (1H, m), 7.74 (1H, d, $J = 8.2$). **^{13}C NMR** (101 MHz, CDCl_3): 14.25, 21.51, 22.02, 23.06, 23.45, 24.91, 24.97, 27.03, 36.49, 40.77, 43.77, 51.63, 52.04, 53.54, 55.13, 61.76, 171.60, 172.44, 175.74, 194.29. **Optical rotation**: $[\alpha]_D^{22}$ - 25.8° (c 0.124, DMF). **IR** (KBr): 3413 s, vbr, 3314 s, vbr, 3072 m, br, 2105 s, 1739 s, 1655



vs, br, 1539 s, br, 1468 s, 1386 s, 1370 s, 1208 s, br, 1029 s, cm^{-1} . **ESI MS:** 426 ($[\text{M} + \text{H}]^+$). **HR ESI MS:** calcd for $\text{C}_{20}\text{H}_{36}\text{O}_5\text{N}_5$ 426.27110; found 426.27124.

Scheme 5



5

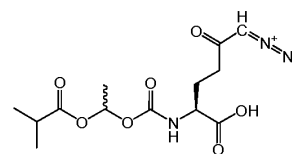
Synthesis of 6-diazo-2-(((1-(isobutyryloxy)ethoxy)carbonyl)amino)-5-oxohexanoic acid triethylammonium salt (29)

6-Diazo-2-(((1-(isobutyryloxy)ethoxy)carbonyl)amino)-5-oxohexanoic acid triethylammonium salt (29). Referring to scheme 5, compound **20** (121 mg, 0.607

10 mmol, 1 eq.) was dissolved in THF (5 mL). 1M solution of NaOH (24 mg, 607 μL , 1 eq.) was added and the mixture was stirred for 1 h. Water (2 mL) and 1-[[[(2,5-

Dioxopyrrolidin-1-yl)oxy]carbonyl]oxy]ethyl 2-

15 methylpropanoate (**LTP 150**) (183 mg, 0.668 mmol, 1.1 eq.) in THF (3 mL) were added. The solution was stirred for next 2 h at rt. The reaction was quenched with 1 M formic acid (607 μL , 1 eq.) and after 5 minutes of stirring the mixture was evaporated to dryness. The crude product was purified by preparative HPLC with $\text{Et}_3\text{N}/\text{CH}_3\text{COOH}$ buffer to give **29** as a light orange solid (106 mg, 53 %). **^1H NMR**



(400 MHz, CDCl₃): 1.11 (6H, d, $J = 6.4$), 1.35 – 1.46 (3H, m), 1.84 – 2.32 (2H, m), 2.36 – 2.75 (2H, m), 4.15 – 4.48 (1H, m), 4.60 – 4.97 (1H, m), 5.70 – 6.12 (1H, m), 6.67 – 6.82 (1H, m), 8.40 (1H, bs).

Synthesis of 6-diazo-2-(((1-(pivaloyloxy)ethoxy)carbonyl)amino)-5-oxohexanoic acid

5 triethylammonium salt (**30**)

6-Diazo-2-(((1-(pivaloyloxy)ethoxy)carbonyl)amino)-5-oxohexanoic acid

triethylammonium salt (30). Referring to scheme 5, compound **20** (89 mg, 0.446

mmol, 1 eq.) was dissolved in THF (3 mL). 1M solution of

NaOH (18 mg, 446 μ L, 1 eq.) was added and the mixture

10 was stirred for 1 h. Water (1 mL) and 1-(((2,5-

Dioxopyrrolidin-1-yl)oxy)carbonyl)oxy)ethyl pivalate

(**LTP174**) (141 mg, 0.490 mmol, 1.1 eq.) in THF (1.5 mL) were added. The solution

was stirred for next 2 h at rt. The reaction was quenched with 1 M formic acid (446

μ L, 1 eq.) and after 5 minutes of stirring the mixture was evaporated to dryness. The

15 crude product was purified by preparative HPLC with Et₃N/CH₃COOH buffer to give

30 as a light orange solid (64 mg) in 42 % yield. ¹H NMR (400 MHz, CDCl₃): 1.18

(9H, s), 1.36 – 1.59 (3H, m), 1.94 – 2.36 (2H, m), 2.40 – 2.77 (2H, m), 4.18 – 4.48

(1H, m), 4.53 – 4.97 (1H, m), 5.45 – 6.07 (1H, m), 6.68 – 6.84 (1H, m), 8.87 (1H, bs).

Synthesis of ethyl 6-diazo-2-(((1-(isobutyryloxy)ethoxy)carbonyl)amino)-5-

20 *oxohexanoate (31)*

Ethyl 6-diazo-2-(((1-(isobutyryloxy)ethoxy)carbonyl)amino)-5-oxohexanoate

(**31**). 1-[[[(2,5-Dioxopyrrolidin-1-yl)oxy]carbonyl]oxy]ethyl 2-methylpropanoate (226

mg, 0.828 mmol, 1.1 eq) was suspended in dry DCM (6

mL). The reaction mixture was cooled to 0 °C and

25 compound **20** (150 mg, 0.753 mmol, 1 eq.) in dry DCM (3

mL) was added by drop wise. The mixture was stirred for

15 minutes at 0 °C and then 1 h at rt. The crude product was purified by column

chromatography (EtOAc/hexane 1:10 to 1:2) and the desired compound **31** was

obtained in 56 % yield (150 mg) as a yellow oil (mixture of two stereoisomers 1:1).

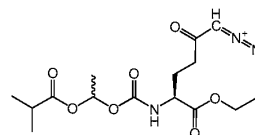
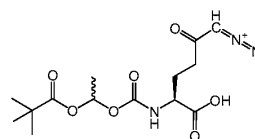
30 ¹H NMR (400 MHz, CDCl₃, Stereoisomer 1): 1.14 (6H, d, $J = 6.7$), 1.27 (3H, t, $J =$

7.1), 1.46 (3H, d, $J = 5.4$), 1.88 – 2.09 (1H, m), 2.16 – 2.29 (1H, m), 2.34 – 2.47 (2H,

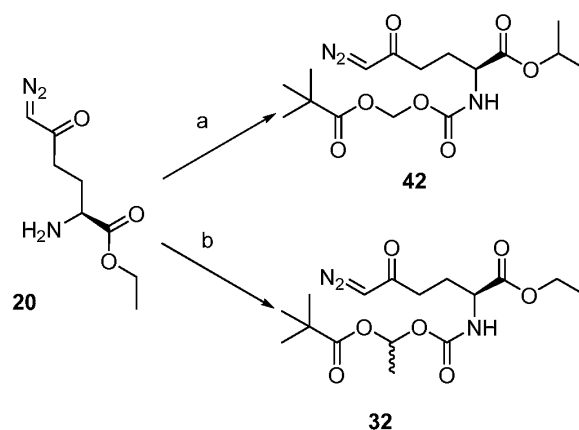
m), 2.51 (1H, sep, $J = 7.1$), 4.19 (2H, q, $J = 7.1$), 4.27 – 4.36 (1H, m), 5.29 (1H, brs),

5.49 (1H, d, $J = 8.3$), 6.78 (1H, q, $J = 7.7$). ¹³C NMR (101 MHz, CDCl₃,

Stereoisomer 1): 14.26, 18.78, 18.80, 19.84, 27.73, 33.99, 36.35, 53.39, 54.92, 61.92,



Scheme 6



15 *Synthesis of ethyl 6-diazo-2-(((1-(pivaloyloxy)ethoxy)carbonyl)amino)-5-oxohexanoate (32)*

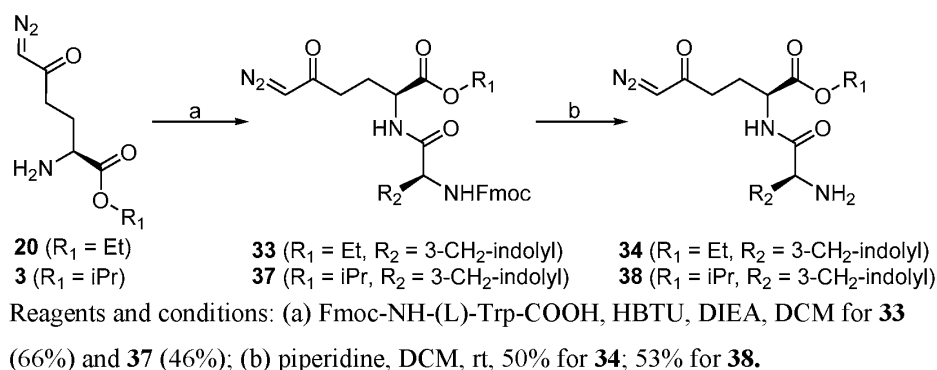
Referring to scheme 6, 1-(((2,5-Dioxopyrrolidin-1-yl)oxy)carbonyl)oxy ethyl pivalate (238 mg, 0.828 mmol,

CCOC(=O)C(=O)[C@H](CC(=O)OCC)NC(=O)OCC(C)(C)C(=O)OCC

0.753 mmol, 1 eq.) in dry DCM (3 mL) was added by drop wise. The mixture was stirred for 15 minutes at 0 °C and then 1 h at rt. The crude product was purified by column chromatography (EtOAc/hexane 1:10 to 1:2) and the desired compound **32**

was obtained in 68 % yield (190 mg) as a yellow oil (mixture of two stereoisomers 1:1). ¹H NMR (400 MHz, CDCl₃, Stereoisomer 1): 1.16 (9H, s), 1.26 (3H, t, *J* = 7.1), 1.43 (3H, s), 1.88 – 2.05 (1H, m), 2.14 – 2.26 (1H, m), 2.30 – 2.50 (2H, m), 4.18 (2H, q, *J* = 7.1), 4.24 – 4.34 (1H, m), 5.31 (1H, brs), 5.51 (1H, d, *J* = 7.7), 6.74 (1H, q, *J* = 7.3). ¹³C NMR (101 MHz, CDCl₃, Stereoisomer 1): 14.23, 19.72, 26.95, 27.57, 36.31, 38.74, 53.33, 54.89, 61.85, 89.66, 153.87, 171.61, 176.56, 193.56. ¹H NMR (400 MHz, CDCl₃, Stereoisomer 2): 1.18 (9H, s), 1.26 (3H, t, *J* = 7.1), 1.45 (3H, s), 1.88 – 2.05 (1H, m), 2.14 – 2.26 (1H, m), 2.30 – 2.50 (2H, m), 4.18 (2H, q, *J* = 7.1), 4.24 – 4.34 (1H, m), 5.33 (1H, brs), 5.55 (1H, d, *J* = 7.9), 6.76 (1H, q, *J* = 7.3). ¹³C NMR (101 MHz, CDCl₃, Stereoisomer 2): 14.24, 19.74, 26.97, 27.80, 36.46, 38.75, 53.48, 54.89, 61.87, 89.81, 153.95, 171.77, 176.68, 193.75. **Optical rotation:** [α]_D²² +16.2° (c 0.259, CHCl₃). **IR** (CHCl₃): 3428 m, 3358 w, 3116 w, 2982 s, 2874 m, 2110 s, 1740 vs, 1640 s, 1510 s, 1480 m, 1393 s (sh), 1377 s, 1349 s, 1284 s, 1232 s, 1025 s, cm⁻¹. **ESI MS:** 394 ([M + Na]⁺). **HR ESI MS:** calcd for C₁₆H₂₅O₇N₃Na 394.15847; found 394.15886.

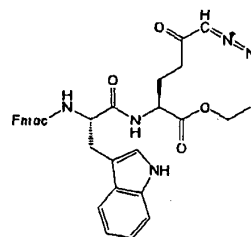
Scheme 7



Synthesis of ethyl 2-(2-amino-3-(1H-indol-3-yl)propanamido)-6-diazo-5-oxohexanoate (34)

Ethyl 2-(2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(1H-indol-3-yl)propanamido)-6-diazo-5-oxohexanoate (33). Referring to scheme 7, Fmoc-NH-(L)-Trp-COOH (353 mg, 0.828 mmol, 1.1 eq.) and HBTU (343 mg, 0.904 mmol, 1.2 eq.) were suspended in dry DCM (5 mL). DIEA (292 mg, 394 μ L, 2.26 mmol, 3 eq.) and then the solution of **20** (150 mg, 0.753 mmol, 1 eq.) in dry DCM (3 mL) were

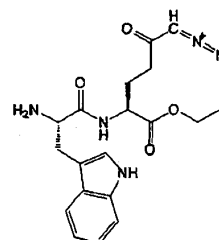
added by syringe. The reaction mixture was stirred for 2 h at rt under inert atmosphere. DCM (20 mL) was added and the organic phase was washed with sat. NaHCO₃ (20 mL), 1M HCl (20 mL), water (30 mL) and sat. NaCl (30 mL),



dried over MgSO₄. DCM was evaporated. The crude product was purified by LC (DCM/EtOAc 2:1, R_f 0.21) to give **33** as a light yellow solid (303 mg) in 66 % yield. ¹H NMR (400 MHz, CDCl₃): 1.22 (3H, t, *J* = 7.2), 1.82 – 1.93 (1H, m), 2.00 – 2.26 (3H, m), 3.18 (1H, dd, *J* = 14.5, 7.1), 3.38 (1H, dd, *J* = 14.0, 5.3), 4.09 (2H, q, *J* = 8.2), 4.20 (1H, t, *J* = 7.1), 4.32 – 4.48 (3H, m), 4.50 – 4.59 (1H, m), 5.05 (1H, bs), 5.51 (1H, d, *J* = 7.9), 6.61 (1H, d, *J* = 7.5), 7.07 (1H, bs), 7.13 (1H, t, *J* = 7.4), 7.20 (1H, t, *J* = 7.2), 7.30 (2H, t, *J* = 7.5), 7.35 (1H, d, *J* = 8.0), 7.40 (2H, t, *J* = 7.5), 7.57 (2H, t, *J* = 6.6), 7.67 (1H, d, *J* = 7.9), 7.76 (2H, d, *J* = 7.6), 8.28 (1H, bs). ¹³C NMR (101 MHz, CDCl₃): 14.20, 27.09, 28.51, 36.20, 47.25, 52.13, 54.86, 55.76, 61.77, 67.25, 110.29, 111.39, 118.88, 120.00, 120.10, 120.11, 122.41, 123.66, 125.25, 125.29, 126.92, 127.22 (2C), 127.62, 127.86 (2C), 136.38, 141.40, 143.87, 143.96, 156.09, 171.28, 171.48, 193.84. **Optical rotation:** [α]_D²² - 28.6° (c 0.178, DMF). **IR** (KBr): 3424 s, 3308 m, sh, 2978 w, 2106 m, 1728 s, 1697 m, sh, 1654 m, 1519 m, 1478 w, 1450 m, 1382 m, sh, 1376 m, 1343 m, 1289 m, 1224 m, 1104 m-w, 1081 m-w, 1040 m, 1032 m, 877 w, 855 w, 760 m, 742 m, 621 m, 427 w, cm⁻¹. **ESI MS:** 630 ([M + Na]⁺). **HR ESI MS:** calcd for C₃₄H₃₃O₆N₅Na 630.23230; found 630.23236.

Ethyl 2-(2-amino-3-(1H-indol-3-yl)propanamido)-6-diazo-5-oxohexanoate (34).

Referring to scheme 7, compound **33** (303 mg, 0.499 mmol, 1 eq.) was dissolved in dry DCM (5 mL). Piperidine (212 mg, 244 μ L, 2.49 mmol, 5 eq.) was added by syringe. The reaction mixture was stirred for 4.5 h at rt under inert atmosphere.



DCM was evaporated. The crude product was purified by column LC (CHCl₃/MeOH 20:1 to 10:1, R_f 0.29) and a colorless solid **34** (96 mg) was obtained in 50 % yield. ¹H NMR (400 MHz, CDCl₃): 1.27 (3H, t, *J* = 7.0), 1.57 (2H, bs), 1.91 – 2.01 (1H, m), 2.06 – 2.27 (3H, m), 3.05 (1H, dd, *J* = 14.4, 8.1), 3.30 (1H, dd, *J* = 14.4, 4.0), 3.75 (1H, dd, *J* = 8.1, 4.2), 4.18 (1H, qd, *J* = 7.1, 2.0), 4.55 (1H, td, *J* = 8.3, 4.1), 5.11 (1H, bs), 7.09 (1H, d, *J* = 2.4), 7.12 (1H, ddd, *J* = 8.1, 7.0, 1.1), 7.20 (1H, ddd, *J* = 8.1, 7.0, 1.2), 7.37 (1H, dt, *J* = 8.2, 1.0), 7.68 (1H, dd, *J* = 7.9, 1.1), 7.88 (1H, d, *J* = 8.3), 8.31 (1H, bs). ¹³C NMR

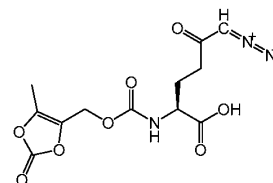
35

(101 MHz, CDCl₃): 14.29, 27.60, 30.82, 36.58, 51.56, 54.77, 55.50, 61.71, 111.36, 111.49, 119.23, 119.79, 122.39, 123.45, 127.69, 136.52, 171.92, 175.02, 193.81.

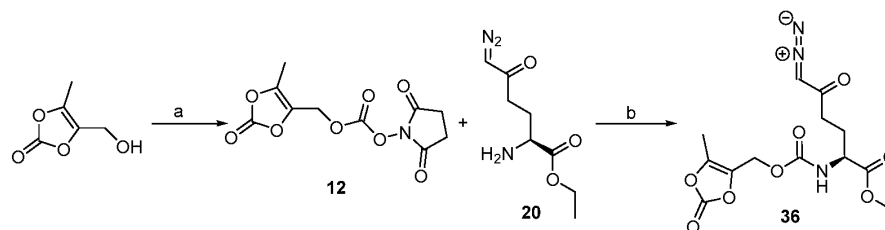
Optical rotation: $[\alpha]_D^{22}$ - 57.6° (c 0.210, DMF). **IR** (CHCl₃): 3479 m, 3355 w, 3215 vw, 3116 w, 2982 m, 2930 m, 2872 w, 2855 w, 2110 vs, 1737 s, 1687 s, 1641 m, 1511 m, 1373 m, 1353 m, 1336 m, sh, 1191 m, 1115 w, 1092 m, 1009 w, cm⁻¹. **ESI MS:** 408 ([M + Na]⁺). **HR ESI MS:** calcd for C₁₉H₂₃O₄N₅Na 408.16423; found 408.16435.

Synthesis of 6-diazo-2-((((5-methyl-2-oxo-1,3-dioxol-4-yl)methoxy)carbonyl)amino)-5-oxohexanoic acid triethylammonium salt (35)

- 10 **6-Diazo-2-((((5-methyl-2-oxo-1,3-dioxol-4-yl)methoxy)carbonyl)amino)-5-oxohexanoic acid triethylammonium salt (35).** Referring to scheme 5, compound **20** (238 mg, 1.19 mmol, 1 eq.) was dissolved in THF (10 mL). 1M solution of NaOH (48 mg, 1.18 mL, 1 eq.) was added and the mixture was stirred for 1 h. Water (4 mL)
- 15 and **12** (389 mg, 1.43 mmol, 1.2 eq.) in THF (10 mL) were added. The solution was stirred for next 2 h at rt. The reaction was quenched with 1 M formic acid (1.15 mL, 0.95 eq.) and after 5 minutes of stirring the mixture was evaporated to dryness. The crude product was purified by preparative HPLC with Et₃N/CH₃COOH buffer. The desired product **35** was obtained
- 20 as a light orange solid (50 mg) in 13 % yield. **¹H NMR** (400 MHz, CDCl₃): 1.96 – 2.28 (2H, m), 2.15 (3H, s), 2.37 – 2.70 (2H, m), 4.40 – 4.76 (1H, m), 4.98 (2H, s).



Scheme 8

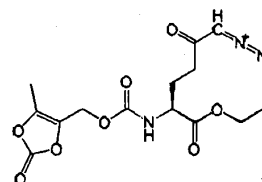


- 25 Reagents and conditions: (a) *S*-ethyl carbonochloridothionate, pyridine, Et₂O; NHS, peracetic acid, DCM 58%; (b) DCM, 0 °C-rt, 65%

Synthesis of ethyl 6-diazo-2-((((5-methyl-2-oxo-1,3-dioxol-4-yl)methoxy)carbonyl)amino)-5-oxohexanoate (36)

Ethyl 6-diazo-2-(((5-methyl-2-oxo-1,3-dioxol-4-yl)methoxy)carbonyl)amino)-5-oxohexanoate (36). Referring to scheme 8, compound **12** (180 mg, 0.663 mmol, 1.1 eq) was suspended in dry DCM (5 mL). The reaction mixture was cooled to 0 °C and compound **20** (120 mg,

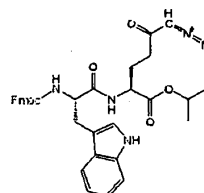
5 0.602 mmol, 1 eq.) in dry DCM (3 mL) was added by drop wise. The mixture was stirred for 15 minutes at 0 °C and then 1 h at rt. The crude product was purified by



column chromatography (EtOAc : hexane, 1 : 1) and the desired compound **36** was obtained in 65 % yield (139 mg) as an yellow oil. ¹H NMR (400 MHz, CDCl₃): 1.27 (3H, t, *J* = 7.1), 1.95–2.06 (1H, m), 2.10–2.27 (1H, m), 2.16 (3H, s), 2.31–2.50 (2H, m), 4.19 (2H, q, *J* = 7.1), 4.29 (1H, td, *J* = 8.1, 4.8), 4.82 (2H, dd, *J* = 13.9), 5.29 (1H, brs), 5.72 (1H, d, *J* = 7.7). ¹³C NMR (101 MHz, CDCl₃): 9.48, 14.24, 27.18, 36.32, 53.76, 54.58, 55.00, 61.92, 133.90, 140.08, 152.30, 155.26, 171.63, 193.52. **Optical rotation:** [α]_D²² + 12.2° (c 0.229, CHCl₃). **IR** (CHCl₃): 3425 m, 3345 w, vbr, 3116 w, 2111 vs, 1838 s, sh, 1820 vs, 1736 vs, 1722 vs, sh, 1640 s, 1511 s, 1229 s, 1200 s, 1114 m, 1097 m, 1045 s, cm⁻¹. **ESI MS:** 378 ([M + Na]⁺). **HR ESI MS:** calcd for C₁₄H₁₇O₈N₃Na 378.09079; found 378.09102.

Synthesis of isopropyl 2-((2-amino-3-(1H-indol-3-yl)propanamido)-6-diazo-5-oxohexanoate (38)

Isopropyl 2-((2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(1H-indol-3-yl)propanamido)-6-diazo-5-oxohexanoate (37). Referring to scheme 7, Fmoc-NH-(*L*)-Tyr-COOH (880 mg, 2.06 mmol, 1.1 eq.) and HBTU (854 mg, 2.25 mmol, 1.2 eq.) were suspended in dry DMF (14 mL). DIEA (727 mg, 980 μL, 5.63 mmol, 3 eq.) and then the solution of **3** (400 mg, 1.88 mmol, 1 eq.) in dry DMF (5 mL) were added by syringe. The reaction mixture was stirred

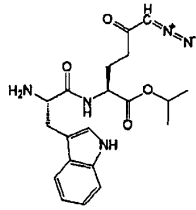


for 4 h at rt under inert atmosphere. DMF was evaporated, DCM (100 mL) was added and the organic phase was washed with sat. NaHCO₃ (100 mL), water (100 mL), 1M HCl (100 mL), water (100 mL) and sat. NaCl (100 mL), dried over MgSO₄. DCM was evaporated. The crude product was purified by column chromatography (DCM:EtOAc, 5:1, R_f 0.15) to give **37** as a light yellow solid (540 mg) in 46 % yield. ¹H NMR (400 MHz, CDCl₃): 1.19 (3H, d, *J* = 6.3), 1.23 (3H, t, *J* = 6.3), 1.81–1.92 (1H, m), 2.01–2.28 (3H, m), 3.18 (1H, dd, *J* = 14.5, 7.1), 3.39 (1H, dd, *J* = 14.2,

35

5.2), 4.20 (1H, t, $J = 7.1$), 4.30 – 4.48 (3H, m), 4.54 (1H, q, $J = 6.9$), 4.88 – 4.99 (1H, m), 5.07 (1H, bs), 5.50 (1H, d, $J = 7.9$), 6.59 (1H, d, $J = 7.4$), 7.07 (1H, bs), 7.14 (1H, t, $J = 7.0$), 7.20 (1H, t, $J = 7.2$), 7.30 (2H, tdd, $J = 7.5, 2.5, 1.1$), 7.36 (1H, d, $J = 8.0$), 7.40 (2H, t, $J = 7.5$), 7.56 (2H, t, $J = 6.6$), 7.67 (1H, d, $J = 7.9$), 7.77 (2H, d, $J = 7.6$), 8.23 (1H, bs). ^{13}C NMR (101 MHz, CDCl_3): 21.78, 21.83, 27.20, 28.47, 36.20, 47.23, 52.19, 54.91, 55.75, 67.23, 69.22, 110.28, 111.40, 118.88, 120.03, 120.10, 120.12 (2C), 122.45, 123.66, 125.25, 125.30, 127.23 (2C), 127.61, 127.86 (2C), 136.35, 141.40 (2C), 143.85, 143.96, 156.09, 170.80, 171.42, 193.92. **Optical rotation:** $[\alpha]_D^{22} - 32.0^\circ$ (c 0.193, DMF). **IR** (KBr): 3424 s, 3300 m, 3130 vw, 2980 w, 2932 s, 2110 m, 1722 s, 1695 s, 1654 s, 1625 m, 1547 m, sh, 1532 m, 1520 m, sh, 1478 w, 1385 m, 1375 m, 1353 m, 1343 m, 1288 m, 1236 m, 1182 w, 11145 m, 1105 m, 1031 m-w, 1010 w, 852 w, 798 w, 758 m, 741 m, 621 w, 427 w-m, cm^{-1} . **ESI MS:** 644 ($[\text{M} + \text{Na}]^+$). **HR ESI MS:** calcd for $\text{C}_{35}\text{H}_{35}\text{O}_6\text{N}_5\text{Na}$ 644.24795; found 644.24811.

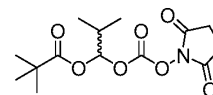
Isopropyl 2-(2-amino-3-(1H-indol-3-yl)propanamido)-6-diazo-5-oxohexanoate

15 (38). Referring to scheme 7, compound 37 (500 mg, 0.804 mmol, 1 eq.) was dissolved in dry DCM (15 mL). Piperidine (342 mg, 393 μL , 4.02 mmol, 5 eq.) was added by syringe. The reaction mixture was stirred for 4 h at rt under inert atmosphere. DCM was evaporated. The crude product was purified by column LC

 20 ($\text{CHCl}_3/\text{MeOH}$ 30:1, R_f 0.14) to give 38 as a yellow amorphous solid (170 mg) in 53 % yield. ^1H NMR (400 MHz, CDCl_3): 1.24 (3H, d, $J = 6.0$), 1.25 (3H, d, $J = 6.0$), 1.50 (2H, bs), 1.88 – 2.00 (1H, m), 2.04 – 2.27 (3H, m), 3.04 (1H, dd, $J = 14.4, 8.2$), 3.30 (1H, ddd, $J = 14.2, 4.2, 0.9$), 3.74 (1H, dd, $J = 8.1, 4.2$), 4.51 (1H, td, $J = 8.3, 4.0$), 5.02 (1H, hept, $J = 6.0$), 5.11 (1H, bs), 7.08 (1H, d, $J = 2.3$), 7.11
 25 (1H, ddd, $J = 8.0, 7.1, 1.0$), 7.19 (1H, ddd, $J = 8.1, 7.1, 1.1$), 7.36 (1H, d, $J = 8.1$), 7.67 (1H, d, $J = 7.9$), 7.88 (1H, d, $J = 8.2$), 8.45 (1H, bs). ^{13}C NMR (101 MHz, CDCl_3): 21.83, 27.65, 30.81, 36.55, 51.64, 54.80, 55.51, 69.49, 111.37 (2C), 119.17, 119.72, 122.31, 123.48, 127.67, 136.53, 171.42, 175.09, 193.92. **Optical rotation:** $[\alpha]_D^{21} - 1.2^\circ$ (c 0.012, CHCl_3). **IR** (CHCl_3): 3311 m, vbr, 2980 w, 2924 w, 2874 w, vs, 2853 w, 2104 vs, 1731 s, 1650 s, br, 1618 m, sh, 1512 m, 1388 m, sh, 1375 s, 1253 m, 1232 m, 1183 m, 1145 m, 1105 s, 1010 w, 972 vw, 933 vw, 744 m, cm^{-1} . **ESI MS:** 422 ($[\text{M} + \text{Na}]^+$). **HR ESI MS:** calcd for $\text{C}_{20}\text{H}_{25}\text{O}_4\text{N}_5\text{Na}$ 422.17988; found 422.17992.

Synthesis of isopropyl 6-diazo-2-(((2-methyl-1-(pivaloyloxy)propoxy)carbonyl)amino)-5-oxohexanoate (40)

1-(((2,5-Dioxopyrrolidin-1-yl)oxy)carbonyl)oxy)-2-methylpropyl pivalate (39). 1-

- Chloro-2-methylpropyl carbonochloridate (2.00 g, 1.71 mL, 11.69 mmol, 1 eq.) was dissolved in dry Et₂O (20 mL). The reaction mixture was cooled to 0 °C and the mix of Et₃N (1.18 g, 1.63 mL, 11.69 mmol, 1 eq.) and EtSH (727 mg, 866 µL, 11.69 mmol, 1 eq.) dissolved in dry Et₂O (10 mL) was added by dropwise method during 10 minutes. The reaction mixture was stirred overnight (23 h) at rt, precipitate was filtered over pad of celite and solvent was removed under reduced pressure. The crude product *O*-(1-chloro-2-methylpropyl) *S*-ethyl carbonothioate (colorless liquid, 2.20 g, 96%) was used for further step without purification. *O*-(1-Chloro-2-methylpropyl) *S*-ethyl carbonothioate (1.20 g, 6.10 mmol, 1 eq.) was dissolved in pivalic acid (3.74 g, 4.20 mL, 36.61 mmol, 6 eq.) and freshly prepared salt of pivalic acid (1.87 g, 2.10 mL, 18.31 mmol, 3 eq.) and DIEA (2.37 g, 3.19 mL, 18.31 mmol, 3 eq.) was added in few portions. The reaction mixture was heated to 60 °C for 70 h. EtOAc (100 mL) was added and the organic phase was extracted with water (50 mL), sat. NaHCO₃ (3×50 mL), sat. NaCl (50 mL), dried over MgSO₄ and solvent was evaporated. The crude product 1-(((ethylthio)carbonyl)oxy)-2-methylpropyl pivalate (light yellow liquid, 1.32 g, 83 %) was used for further step without purification. 1-(((Ethylthio)carbonyl)oxy)-2-methylpropyl pivalate (1.28 g, 4.88 mmol, 1 eq.) was dissolved in dry DCM (13 mL), *N*-hydroxysuccinimide (1.12 g, 9.76 mmol, 2 eq.) was added and the suspension was cooled to 0 °C. Peracetic acid (1.11 g (100 %), 3.09 g (36 %), 14.64 mmol, 3 eq., 36 % solution in acetic acid) was added by dropwise in 10 minutes. The final mixture was stirred for 60 minutes at 0 °C and 2 h at rt. DCM (40 mL) was added and the organic phase was washed with water (20 mL) and sat. NaCl (20 mL) and dried over MgSO₄. DCM was evaporated and the product was purified by LC (hexane/EtOAc 5:3, R_f 0.26). The product **39** was obtained as a light yellow oil (863 mg) in 56 % yield (over 3 steps). **¹H NMR** (400 MHz, CDCl₃): 1.00 (6H, d, *J* = 6.9), 1.21 (9H, s), 2.08-2.19 (1H, m), 2.81 (4H, s), 6.55 (1H, d, *J* = 5.0). **¹³C NMR** (101 MHz, CDCl₃): 16.06, 16.38, 25.55, 26.89, 31.84, 39.05, 98.19, 150.37, 168.48, 176.14. **Optical rotation:** [α]_D²² - 3.0° (c 0.230, CHCl₃). **IR** (CHCl₃): 2978 m, 2938 w, 2878 w, 1821 s, 1795 s, 1748 vs, br, 1481 m, 1463 w, 1432 m, 1396 w, 1373 m, 1366 m, sh, 1279 m, 1199 s, 1046 m, 998 m, sh, 987 m, 932



s cm⁻¹. **ESI MS**: 338 ([M + Na]⁺). **HR ESI MS**: calcd for C₁₄H₂₁O₇NNa 338.12102; found 338.12115.

Isopropyl 6-diazo-2-(((2-methyl-1-(pivaloyloxy)propoxy)carbonyl)amino)-5-

oxohexanoate (40). Compound **39** (399 mg, 1.27 mmol, 0.9 eq) was suspended in dry

5 DCM (7 mL). The reaction mixture was cooled to 0 °C

and compound **3** (300 mg, 1.41 mmol, 1 eq.) in dry

DCM (3 mL) was added by drop wise. The mixture was

stirred for 15 minutes at 0 °C and then 2 h at rt. The

crude product was purified by column chromatography (EtOAc/hexane 1:2, R_f 0.29

10 and 0.31) and the desired compound **40** was obtained in 54 % yield (285 mg) as a

yellow oil (mixture of two stereoisomers 1:1). **¹H NMR** (400 MHz, CDCl₃, stereoisomer 1): 0.94 (6H, d, *J* = 6.8), 1.16 (9H, s), 1.23 (6H, t, *J* = 6.3), 1.83 – 2.50 (4H, m), 4.22 – 4.31 (1H, m), 5.02 (1H, hept, *J* = 6.8), 5.29 (1H, bs), 5.48 (1H, d, *J* = 8.3), 6.52 (1H, d, *J* = 4.9). **¹³C NMR** (101 MHz, CDCl₃, stereoisomer 1): 16.40,

15 16.54, 27.00, 28.05, 31.87, 36.29, 38.96, 53.38, 54.82, 69.64, 94.21, 154.28, 171.31,

176.56, 193.87. **¹H NMR** (400 MHz, CDCl₃, stereoisomer 2): 0.93 (6H, d, *J* = 6.8), 1.18 (9H, s), 1.22 (6H, t, *J* = 6.3), 1.83 – 2.50 (4H, m), 4.22 – 4.31 (1H, m), 5.00 (1H, sept, *J* = 6.8), 5.37 (1H, bs), 5.45 (1H, d, *J* = 8.3), 6.48 (1H, d, *J* = 4.9). **¹³C NMR**

20 53.54, 54.82, 69.66, 93.87, 154.22, 171.17, 176.81, 193.58. **Optical rotation**: [α]_D²² +

11.5° (c 0.261, CHCl₃). **IR** (CHCl₃): 3428 m, 3116 w, 2982 s, 2936 m, 2878 m, 2110 vs, 1741 vs, br, 1731 vs, sh, 1641 s, 1508 s, 1480 m, 1463 m, 1400 m, sh, 1385 s, sh, 1377 s, 1365 s, sh, 1281 s, 1231 s, 1183 m, 1146 s, 1105 s, 990 s, 941 m cm⁻¹. **ESI**

MS: 436 ([M + Na]⁺). **HR ESI MS**: calcd for C₁₉H₃₁O₇N₃Na 436.20542; found

25 436.20553.

Synthesis of isopropyl 6-diazo-5-oxo-2-

(((pivaloyloxy)methoxy)carbonyl)amino)hexanoate (42)

(((2,5-Dioxopyrrolidin-1-yl)oxy)carbonyl)oxy)methyl pivalate (41). Chloromethyl carbonochloridate (2.00 g, 1.38 mL, 15.51 mmol, 1 eq.) was dissolved in dry Et₂O (20

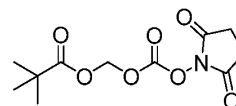
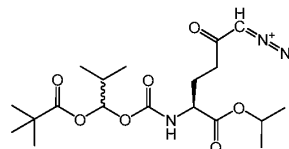
30 mL). The reaction mixture was cooled to 0 °C and the mix of

Et₃N (1.57 g, 2.16 mL, 15.51 mmol, 1 eq.) and EtSH (964

mg, 1.15 mL, 15.51 mmol, 1 eq.) dissolved in dry Et₂O (5

mL) was added by dropwise method during 5 minutes. The reaction mixture was

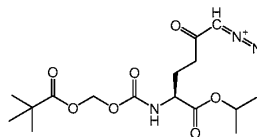
stirred overnight (25 h) at rt, precipitate was filtered over pad of celite and solvent



was removed under reduced pressure. The crude product *O*-(chloromethyl) *S*-ethyl carbonothioate (colorless liquid) was used for further step without purification. *O*-(Chloromethyl) *S*-ethyl carbonothioate (2.40 g, 15.51 mmol, 1 eq.) was dissolved in pivalic acid (9.51 g, 93.14 mmol, 6 eq.) and freshly prepared salt of pivalic acid (4.76 g, 46.57 mmol, 3 eq.) and DIEA (6.02 g, 8.1 mL, 46.57 mmol, 3 eq.) was added in few portions. The reaction mixture was heated to 60 °C for 22 h. EtOAc (100 mL) was added and the organic phase was extracted with water (100 mL), sat. NaHCO₃ (3×100 mL), sat. NaCl (100 mL), dried over MgSO₄ and solvent was evaporated. The crude product (((ethylthio)carbonyl)oxy)methyl pivalate (light yellow liquid, 3.30 g, 97 %) was used for further step without purification. (((Ethylthio)carbonyl)oxy)methyl pivalate (3.20 g, 14.53 mmol, 1 eq.) was dissolved in dry DCM (40 mL), *N*-hydroxysuccinimide (3.34 g, 29.05 mmol, 2 eq.) was added and the suspension was cooled to 0 °C. Peracetic acid (3.31 g (100 %), 9.21 g (36 %), 43.58 mmol, 3 eq., 36 % solution in acetic acid) was added by dropwise in 15 minutes. The final mixture was stirred for 60 minutes at 0 °C and 2 h at rt. DCM (50 mL) was added and the organic phase was washed with water (30 mL) and sat. NaCl (30 mL) and dried over MgSO₄. DCM was evaporated and the product was purified by LC (hexane : EtOAc, 2 : 1, R_f 0.27). The product **41** was obtained as a colorless solid (2.54 g) in 64 % yield (over 3 steps). ¹H NMR (400 MHz, CDCl₃): 1.24 (9H, s), 2.84 (4H, s), 5.86 (2H, s). ¹³C NMR (101 MHz, CDCl₃): 25.56 (2C), 26.86 (3C), 38.96, 83.67, 150.90, 168.34 (2C), 176.54. IR (CHCl₃): 2979 m, 2939 w, 2876 w, 1823 s, 1796 vs, 1649 vs, 1481 m, 1463 m, 1456 w, 1431 m, 1398 w, 1371 m, 1367 m, 1280 m, 1199 vs, 1110 vs, 1047 m, 998 s, 986 s, 942 m, sh, 924 s, 853 w, cm⁻¹. ESI MS: 296 ([M + Na]⁺). HR ESI MS: calcd for C₁₁H₁₅O₇NNa 296.07407; found 296.07410.

25 Isopropyl 6-diazo-5-oxo-2-(((pivaloyloxy)methoxy)carbonyl)amino)hexanoate

(42). Referring to scheme 6, compound **41** (320 mg, 1.17 mmol, 1.0 eq) was suspended in dry DCM (6 mL). The reaction mixture was cooled to 0 °C and NH₂-DON-COOEt (250 mg, 1.17 mmol, 1 eq.) in dry DCM (3 mL) was added by drop wise.



30 The mixture was stirred for 15 minutes at 0 °C and then 2 h at rt. The crude product was purified by column chromatography (EtOAc:hexane, 1:2, R_f 0.21) and the desired compound **42** was obtained in 40 % yield (175 mg) as a yellow oil. ¹H NMR (400 MHz, CDCl₃): 1.19 (9H, s), 1.23 (3H, d, *J* = 6.2), 1.24 (3H, d, *J* = 6.2), 1.90 – 2.05 (1H, m), 2.14 – 2.25 (1H, m), 2.31 – 2.51 (2H, m), 4.28 (1H,

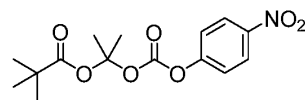
td, $J = 8.2, 4.7$), 5.03 (1H, hept, $J = 6.2$), 5.27 (1H, bs), 5.65 (1H, d, $J = 8.1$), 5.69 (1H, d, $J = 5.7$), 5.73 (1H, d, $J = 5.7$). ^{13}C NMR (101 MHz, CDCl_3): 21.79, 21.81, 26.97, 36.34, 38.86, 53.63, 54.90, 69.75, 80.33, 154.40, 171.01, 177.51, 193.43.

Optical rotation: $[\alpha]_{\text{D}}^{22} + 13.0^\circ$ (c 0.184, CHCl_3). **IR** (CHCl_3): 3424 m, 3354 w, br, 3116 w, 2984 s, 2937 m, 2875 s, 2110 vs, 1747 vs, 1730 vs, sh, 1642 s, 1512 s, 1481 m, 1466 m, 1453 m, 1377 s, 1282 s, 1182 m, 1145 s, 1105 s, 994 s, 942 m, cm^{-1} . **ESI MS:** 394 ($[\text{M} + \text{Na}]^+$). **HR ESI MS:** calcd for $\text{C}_{16}\text{H}_{25}\text{O}_7\text{N}_3\text{Na}$ 394.15847; found 394.15855.

Synthesis of isopropyl 6-diazo-5-oxo-2-(((2-(pivaloyloxy)propan-2-

yl)oxy)carbonyl)amino) hexanoate (44)

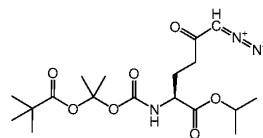
2-(((4-Nitrophenyloxy)carbonyl)oxy)propan-2-yl pivalate (43). 2-Chloropropan-2-yl (4-nitrophenyl) carbonate (prepared by a method reported in US 2006/229361 A1, 300 mg, 1.16 mmol, 1 eq.) was dissolved in dry DCM (15 mL). Mercury pivalate (559 mg, 1.39 mmol, 1.2 eq.) was added and reaction mixture was stirred overnight (19 h) at rt under inert. The solid precipitate (HgCl_2) was removed by filtration, DCM (15 mL) was added and RM was extracted with sat. NaHCO_3 (15 mL), sat brine



(15 mL), dried over MgSO_4 and solvent was evaporated. Product **43** was obtained as a light yellow oil (301 mg) in 80% yield. ^1H NMR (400 MHz, CDCl_3): 1.20 (9H, s), 1.91 (6H, s), 7.34–7.40 (2H, m), 8.24–8.30 (2H, m). ^{13}C NMR (101 MHz, CDCl_3): 25.28 (2C), 27.02 (3C), 39.59, 107.71, 121.98 (2C), 125.40 (2C), 145.50, 149.07, 155.41, 175.97. **IR** (CHCl_3): 3031 w, 2976 w, 2875 w, 1777 m, 1736 m, 1618 w, 1595 w, 1528 m-s, 1493 m, 1481 w, 1439 w, 1396 w, 1376 w, 1349 m, 1322 w, 1264 m, 1191 m, 1112 vs, 1094 s, sh, 1030 w, 980 w, 859 m, 682 vw, 491 vw cm^{-1} . **ESI MS:** 348 ($[\text{M} + \text{Na}]^+$). **HR ESI MS:** calcd for $\text{C}_{15}\text{H}_{19}\text{O}_7\text{NNa}$ 348.10537; found 348.10543.

Isopropyl 6-diazo-5-oxo-2-(((2-(pivaloyloxy)propan-2-yl)oxy)carbonyl)amino) hexanoate (44). Compound **43**

(48 mg, 0.148 mmol, 1 eq.) was dissolved in dry DMF (2 mL) and reaction mixture was cooled to 0 °C. Compound **3**



(79 mg, 0.369 mmol, 2.5 eq.) dissolved in dry DMF (1 mL) was added by syringe. Reaction mixture was stirred at 0 °C under inert overnight for 3 h. DMF was evaporated and the crude mixture was purified by LC (hexane : EtOAc, 2 : 1). Product **44** was obtained as a light yellow oil (49 mg) in 83 % yield. ^1H NMR (400 MHz,

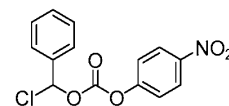
CDCl₃): 1.19 (9H, s), 1.23 (3H, d, $J = 6.3$), 1.24 (3H, d, $J = 6.3$), 1.80 (3H, s), 1.83 (3H, s), 1.90 – 2.01 (1H, m), 2.14 – 2.27 (1H, m), 2.29 – 2.51 (2H, m), 4.24 (1H, dt, $J = 8.3, 4.7$), 5.05 (1H, hept, $J = 6.3$), 5.31 (1H, bs), 5.44 (1H, d, $J = 8.2$). **¹³C NMR** (101 MHz, CDCl₃): 21.80, 21.82, 25.76, 25.91, 27.07 (3C), 27.79, 36.46, 39.48, 53.26, 54.87, 69.59, 105.44, 153.16, 171.31, 176.21, 193.63. **Optical rotation:** $[\alpha]^{22}_{\text{D}} + 12.8^\circ$ (c 0.133, CHCl₃). **IR** (CHCl₃): 3430 w, 3116 w, 2984 m, 2936 m, 2874 m, 2110 s, 1732 vs, br, 1641 m, 1502 s, 1481 m, 1466 m, 1462 m, 1455 m, 1452 m, 1397 m, sh, 1384 s, 1374 s, 1365 s, sh, 1198 s, 1184 s, 1147 m, sh, 1128 s, 1112 s, 1105 s, 1045 m, 942 w cm⁻¹.

10 *Synthesis of isopropyl 6-diazo-5-oxo-2-*

((phenyl(pivaloyloxy)methoxy)carbonyl)amino) hexanoate (47)

Chloro(phenyl)methyl (4-nitrophenyl) carbonate (45). Chloro(phenyl)methyl carbonochloridate (prepared by US20110319422, 900 mg, 4.39 mmol, 1 eq.) was dissolved in dry DCM (20 mL). 4-Nitrophenol (611 mg, 4.39 mmol, 1 eq.) was added

15 and the mixture was cooled to 0 °C. Pyridine (347 mg, 355 μL, 4.39 mmol, 1 eq.) dissolved in dry DCM (5 mL) was added by dropwise method during 5 minutes. Reaction mixture was



stirred for 2 h at rt. DCM was evaporated and the crude product was purified by LC (DCM:hexane, 1:1). The product **45** was obtained as a colorless solid (520 mg) in 39

20 % yield. **¹H NMR** (400 MHz, CDCl₃): 7.33 (1H, s), 7.41–7.50 (5H, m), 7.58–7.63 (2H, m), 8.28–8.34 (2H, m). **¹³C NMR** (101 MHz, CDCl₃): 87.37, 121.83 (2C), 125.59 (2C), 126.41 (2C), 129.07 (2C), 130.56, 136.35, 145.91, 150.50, 155.07.

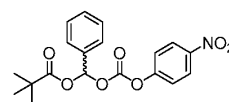
Optical rotation: $[\alpha]^{22}_{\text{D}} - 0.9^\circ$ (c 0.318, CHCl₃). **IR** (CHCl₃): 3119 w, 3088 w, 3071 vw, 3032 w, 1788 vs, 1772 s, sh, 1619 m, 1595 m, 1530 vs, 1492 s, 1456 m, 1349 vs, 1317 m, 1296 m, 1232 vs, sh, 1178 m, sh, 1165 m, 1111 m, 1105 w, sh, 1078 m, 1054 s, 1029 m, 1014 m, 1002 w, 978 s, 920 w, 872 s, 854 s, 830 vw, 708 s, 695 m, sh, 680 w, 626 vw, 618 vw, 530 vw, 495 w, 403 w cm⁻¹. **ESI MS:** 329 ([M + Na]⁺). **HR ESI MS:** calcd for C₁₄H₁₀O₅NCINa 330.01397; found 330.01367.

(((4-Nitrophenyloxy)carbonyl)oxy)(phenyl)methyl pivalate (46). Compound **45**

30 (100 mg, 0.325 mmol, 1 eq.) and mercury pivalate (157 mg, 0.390 mmol, 1.2 eq.) were dissolved in dry DCM (6 mL).

Reaction mixture was stirred at rt under inert overnight (16 h).

DCM (10 mL) was added and reaction mixture was washed with sat. NaHCO₃ (10 mL) and brine (10 mL), organic phase was dried over MgSO₄ and DCM was



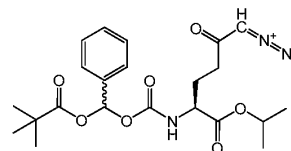
evaporated. The product **46** (115 mg) was obtained in 95 % yield and was used for further step without any purification. ¹H NMR (400 MHz, CDCl₃): 1.28 (9H, s), 7.38–7.43 (2H, m), 7.44–7.50 (3H, m), 7.57–7.60 (1H, m), 7.61 (1H, s), 8.23–8.33 (2H, m). ¹³C NMR (101 MHz, CDCl₃): 27.02 (3C), 39.11, 93.80, 121.86 (2C), 125.47 (2C), 126.84 (2C), 128.97 (2C), 130.48, 134.39, 145.69, 150.73, 155.32, 176.44.

Optical rotation: $[\alpha]^{22}_{\text{D}} - 6.0^{\circ}$ (c 0.201, CHCl₃). **IR** (CHCl₃): 3118 w, 3087 w, 3072 w, 3031 m, 2980 m, 2875 w, 1775 vs, 1747 s, 1618 m, 1595 m, 1529 vs, 1493 s, 1480 m, 1459 m, 1399 m, 1365 m, 1349 vs, 1279 vs, 1248 vs, 1165 s, 1123 vs, 1112 s, sh, 1030 s, 1014 m, 1003 m, 970 s, br, 943 s, 918 m, 865 s, 860 s, 832 w, 697 s, 682 w, 633 w, 619 vw, 530 vw, 495 w, 403 vw cm⁻¹. **ESI MS:** 396 ([M + Na]⁺). **HR ESI MS:** calcd for C₁₉H₁₉O₇NNa 396.10537; found 396.10546.

Isopropyl 6-diazo-5-oxo-2-(((phenyl(pivaloyloxy)methoxy)carbonyl)amino)

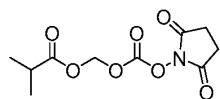
hexanoate (47). Compound **46** (115 mg, 0.308 mmol, 1 eq.) was dissolved in dry DCM (3 mL). DON iPr ester (72 mg, 0.339 mmol, 1.1 eq.) dissolved in dry DCM (2

mL) was added by syringe. Reaction mixture was stirred at rt under inert overnight (24 h) under inert. Further DONiPr ester (72 mg, 0.339 mmol, 1.1 eq.) dissolved in dry DCM (2 mL) was added and stirring was continued



for next 24 h. DCM was evaporated and the crude mixture was purified by preparative HPLC (AcN/H₂O, HCOOH). Product **47** was obtained as a light brown oil (66 mg) in 48 % yield. ¹H NMR (400 MHz, CDCl₃): 1.17 – 1.31 (15H, m), 1.90 – 2.06 (1H, m), 2.12 – 2.31 (1H, m), 2.31 – 2.54 (2H, m), 4.27 – 4.36 (1H, m), 5.03 (1H, hept, *J* = 6.3), 5.29 (1H, bs), 5.59 (1H, d, *J* = 8.1), 7.36 – 7.42 (3H, m), 7.46 – 7.51 (2H, m), 7.61 (s, 1H). ¹³C NMR (101 MHz, CDCl₃): 21.82 (2C), 26.99 (3C), 27.70, 36.35, 38.99, 53.55, 54.90, 69.79, 90.93, 126.57 (2C), 128.66 (2C), 129.64, 135.95, 153.82, 171.10, 176.33, 193.51. **Optical rotation:** $[\alpha]^{22}_{\text{D}} + 12.5^{\circ}$ (c 0.246, CHCl₃). **IR** (CHCl₃): 3425 w, 3116 w, 3098 vw, 3070 vw, 3029 m, 2984 m, 2937 m, 2875 w, 2110 s, 1735 vs, br, 1641 s, 1590 w, 1507 s, 1480 m, 1457 m, 1398 m, 1377 s, 1367 s, sh, 1366 s, sh, 1280 s, 1182 m, 1146 s, sh, 1133 s, 1105 s, 1085 m, 1057 s, 1027 s, 1003 m, 942 m, 918 w, 697 m, 619 vw cm⁻¹. **ESI MS:** 470 ([M + Na]⁺).

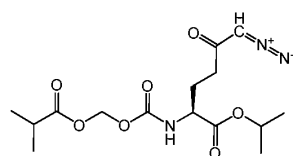
Synthesis of isopropyl 6-diazo-2-(((isobutyryloxy)methoxy)carbonyl)amino)-5-oxohexanoate (49)

(((2,5-Dioxopyrrolidin-1-yl)oxy)carbonyl)oxy)methyl isobutyrate (48).

Chloromethyl carbonochloridate (1.00 g, 690 μ L, 7.76 mmol, 1 eq.) was dissolved in dry Et₂O (10 mL). The reaction mixture was cooled to 0 $^{\circ}$ C and a mixture of Et₃N (785 mg, 1081 μ L,

- 5 7.76 mmol, 1 eq.) and EtSH (482 mg, 574 μ L, 7.76 mmol, 1 eq.) dissolved in dry Et₂O (3 mL) was added by dropwise method over 5 minutes. The reaction mixture was stirred overnight (18 h) at rt, precipitate was filtered over pad of celite and solvent was removed under reduced pressure. The crude product -(chloromethyl) *S*-ethyl carbonothioate (colorless liquid) was used for further step without purification.
- 10 *O*-(Chloromethyl) *S*-ethyl carbonothioate (1.10 g, 7.11 mmol, 1 eq.) was dissolved in isobutyric acid (1.88 g, 1.94 mL, 21.35 mmol, 3 eq.) and freshly prepared salt of isobutyric acid (1.88 g, 1.94 mL, 21.35 mmol, 3 eq.) and DIEA (2.76 g, 3.81 mL, 21.35 mmol, 3 eq.) was added in few portions. The reaction mixture was heated to 60 $^{\circ}$ C for 20 h. EtOAc (50 mL) was added and the organic phase was extracted with
- 15 water (50 mL), sat. NaHCO₃ (3 $\times$ 50 mL), sat. NaCl (50 mL), dried over MgSO₄ and solvent was evaporated. The crude product (((ethylthio)carbonyl)oxy)methyl isobutyrate (light yellow liquid, 1.14 g, 72 %) was used for further step without purification. (((Ethylthio)carbonyl)oxy)methyl isobutyrate (1.12 g, 5.04 mmol, 1 eq.) was dissolved in dry DCM (15 mL), *N*-hydroxysuccinimide (1.16 g, 10.08 mmol, 2
- 20 eq.) was added and the suspension was cooled to 0 $^{\circ}$ C. Peracetic acid (1.15 g (100 %), 3.19 g (36 %), 15.12 mmol, 3 eq., 36 % solution in acetic acid) was added by dropwise in 10 minutes. The final mixture was stirred for 60 minutes at 0 $^{\circ}$ C and 2 h at rt. DCM (20 mL) was added and the organic phase was washed with water (15 mL) and sat. NaCl (15 mL) and dried over MgSO₄. DCM was evaporated and the product
- 25 was purified by LC (hexane:EtOAc, 2:1, R_f 0.24). The product **48** was obtained as a colorless oil (842 mg) in 64 % yield (over 3 steps). ¹H NMR (400 MHz, CDCl₃): 1.19 (6H, d, *J* = 7.0), 2.63 (1H, hept, *J* = 7.0), 2.83 (4H, s), 5.85 (2H, s). ¹³C NMR (101 MHz, CDCl₃): 18.60, 25.54, 33.77, 83.50, 150.92, 168.39, 175.08. IR (CHCl₃): 2981 m, 2945 w, 2880 w, 1823 s, 1795 vs, 1748 vs, br, 1720 m, sh, 1471 m, 1431 m, 1389
- 30 w, 1370 m, 1231 vs, 1199 vs, 1113 s, 1045 m, 925 s, cm⁻¹. ESI MS: 282 ([M + Na]⁺). HR ESI MS: calcd for C₁₀H₁₃O₇NNa 282.05842; found 282.05848.

Isopropyl 6-diazo-2-(((isobutyryloxy)methoxy)carbonyl)amino)-5-oxohexanoate (49). Compound **48** (268 mg, 1.03 mmol, 1.1 equiv) was dissolved in absolute dichloromethane (8 mL). This solution was cooled to 0 $^{\circ}$ C and a solution of the



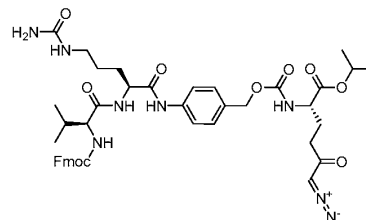
compound **3** (200 mg, 0.94 mmol), in dichloromethane (1 mL) was added dropwise. The reaction mixture was stirred for 15 min at 0 °C in a cooling bath. The reaction mixture was then stirred at room temperature for 1 h.

- 5 The organic solvent was evaporated *in vacuo*. The residue was chromatographed on silica gel (chloroform:acetone, 10:1) to effort the desired product **49** (180 mg, 54 %) as a yellow amorphous solid. **¹H NMR** (400 MHz, CDCl₃): 1.17 – 1.19 (6H, m), 1.25 – 1.27 (6H, m), 1.94 – 2.04 (1H, m), 2.18 – 2.26 (1H, m), 2.32 – 2.49 (2H, m), 2.59 (1H, hept, *J* = 6.9), 4.31 (1H, td, *J* = 8.1, 4.7), 5.05 (1H, hept, *J* = 6.3), 5.28 (1H, s),
 10 5.62 (1H, d, *J* = 8.0), 5.71 (1H, d, *J* = 5.8), 5.75 (1H, d, *J* = 5.8). **¹³C NMR** (101 MHz, CDCl₃): 18.82 (2C), 21.84, 21.86, 27.67, 33.91, 36.37, 53.66, 54.99, 69.84, 80.14, 154.45, 171.03, 176.19. **IR** (CHCl₃): 3424 w, 2111 s, vs, 1750 sh, vs, 1732 vs, 1641 m, s, 1512s, 1387 sh, s, 1377 s, 1370 sh, s cm⁻¹. **Optical rotation**: [α]_D²² + 5.4° (c 0.202, CH₂Cl₂). **ESI MS**: 380 ([M + Na]⁺). **HR ESI MS**: calcd for C₁₅H₂₃O₇N₃Na 380.14282; found 380.14286.

*Synthesis of isopropyl 2-(((4-(2-(2-amino-3-methylbutanamido)-5-ureidopentanamido) benzyl)oxy)carbonyl)amino)-6-diazo-5-oxohexanoate (**51**)*

Isopropyl 2-(((4-(2-(2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)-5-ureidopentanamido)benzyl)oxy)carbonyl)amino)-6-diazo-

- 20 **5-oxohexanoate (**50**)**. 9-Fluorenylmethylloxycarbonyl-valyl-citrullyl-(4-aminobenzyl)-(4-nitrophenyl)carbonate (191 mg, 0.249 mmol, 1.2 equiv.), was dissolved in dry DMF (2.0 mL) and a solution of compound **3** (44 mg, 0.208 mmol) in dry DMF (1.0 mL) was

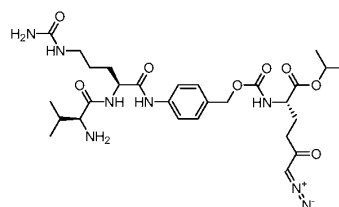


- 25 added dropwise. To this reaction mixture was added diisopropylethyl amine (130 μ L, 0.747 mmol, 3 equiv.) dropwise. The reaction mixture was stirred at room temperature overnight. The organic solvent was evaporated *in vacuo*. The residue was chromatographed on silica gel (chloroform:methanol, 15:1) to yield the desired product **50** (110 mg, 53 %) as a
 30 white amorphous solid. **¹H NMR** (400 MHz, DMSO): 0.85 (3H, d, *J* = 6.8), 0.88 (3H, d, *J* = 6.9), 1.16 (3H, d, *J* = 6.6), 1.18 (3H, d, *J* = 6.6), 1.31 – 1.49 (2H, m), 1.54 – 1.81 (3H, m), 1.89 – 2.03 (2H, m), 2.35 – 2.44 (2H, m), 2.89 – 3.05 (2H, m), 3.91 – 3.98 (2H, m), 4.20 – 4.34 (3H, m), 4.39 – 4.44 (1H, m), 4.86 – 5.00 (3H, m), 5.40 (2H, s), 5.97 (1H, t, *J* = 5.9), 6.05 (1H, s), 7.28 (2H, d, *J* = 8.7), 7.32 (2H, td, *J* = 7.5,

- 1.2), 7.39 – 7.44 (3H, m), 7.59 (2H, d, $J = 8.3$), 7.66 (1H, d, $J = 7.7$), 7.74 (2H, t, $J = 7.8$), 7.89 (2H, d, $J = 7.6$), 8.12 (1H, d, $J = 7.5$), 10.06 (1H, s). ^{13}C NMR (101 MHz, DMSO): 18.28, 19.23, 21.45, 21.52, 25.78, 26.80, 29.48, 30.46, 36.32, 38.58, 46.69, 53.10, 53.42, 60.08, 65.29, 65.69, 68.04, 118.91 (2C), 120.10 (2C), 125.37 (2C), 127.07 (2C), 127.65 (2C), 128.61 (2C), 131.65, 138.64, 140.71 (2C), 143.77, 143.90, 156.13 (2C), 158.90, 170.60, 171.28, 171.55, 194.03. IR (KBr): 3400 s, br, sh, 3327 s, br, 3066 w, 2964 m, 2937 m, 2106 s, 1705 vs, br, 1651 vs, 1609 s, 1533 vs, 1517 vs, sh, 1479 m, 1466 m, 1450 s, 1415 m, 1386 s, sh, 1376 s, 1334 s, 1320 s, sh, 1248 s, 1183 m, 1145 m, 1106 s, 1047 m, 1020 m, sh, 826 w, 777 w, sh, 621 w, 427 w cm^{-1} .
- 10 **Optical rotation:** $[\alpha]_{\text{D}}^{22} - 15.6^\circ$ (c 0.631, DMSO). **ESI MS:** 863 ($[\text{M} + \text{Na}]^+$). **HR ESI MS:** calcd for $\text{C}_{43}\text{H}_{52}\text{N}_8\text{O}_{10}\text{Na}$ 863.36986; found 863.36997.

Isopropyl 2-((((4-(2-(2-amino-3-methylbutanamido)-5-ureidopentanamido) benzyl)oxy)carbonyl)amino)-6-diazo-5-

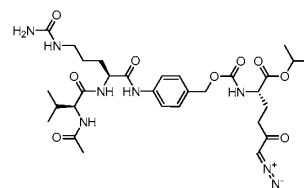
- 15 **oxohexanoate (51).** Compound **50** (110 mg, 0.131 mmol) was dissolved in dry DMF (0.5 mL).



- Piperidine (32 μL 0.327 mmol, 2.5 equiv.) was added and the reaction mixture was stirred at room temperature for 4 h. The organic solvent was evaporated in vacuo. The residue was chromatographed on silica gel (chloroform:methanol, 2:1) to afford the
- 20 desired product **51** (70 mg, 87 %) as a white amorphous solid.

Synthesis of isopropyl 2-((((4-(2-(2-acetamido-3-methylbutanamido)-5-ureidopentanamido) benzyl)oxy)carbonyl)amino)-6-diazo-5-oxohexanoate (52)

- Isopropyl 2-((((4-(2-(2-acetamido-3-methylbutanamido)-5-ureidopentanamido) benzyl)oxy)carbonyl)amino)-6-diazo-5-oxohexanoate (52).** Compound **51** (100 mg, 0.162 mmol), was dissolved in dry DMF (2 mL) and to this solution was added diisopropylethyl amine (144 μL , 0.842 mmol, 5.1 equiv.) was added dropwise followed by acetic anhydride (76.5 μL , 0.81 mmol, 5.0 equiv.). The reaction mixture was stirred at room
- 25 temperature for 2 h. The organic solvent was evaporated *in vacuo*. The residue was chromatographed on silica gel (chloroform:methanol, 7:1) to afford the desired
- 30 product **52** (97 mg, 91 %) as a yellow amorphous solid.



Synthesis of Isopropyl 2-(2-(2-amino-3-methylbutanamido)-5-ureidopentanamido)-6-diazo-5-oxohexanoate (56)

Isopropyl 2-(2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-

ureidopentanamido)-6-diazo-5-oxohexanoate (53). Fmoc-Cit-COOH (2.05 g, 5.16

5 mmol, 1.1 equiv.) and TBTU (1.81 g, 5.63 mmol, 1.2

equiv.) were solved in absolute DMF (40 mL) and

diisopropylethyl amine (2.51 mL, 14.07 mmol, 3 equiv.)

was added. The reaction mixture was stirred at room

temperature for 30 min. and then the solution of **3** (1.0 g, 4.69 mmol) in absolute

10 DMF (20 mL) were added by syringe. The reaction mixture was stirred for 2 h at

room temperature under inert atmosphere. The organic solvent was evaporated in

vacuo. The residue was chromatographed on silica gel (chloroform:methanol, 15:1)

to afford the desired product **53** (1.84 g, 66 %) as a yellow amorphous solid. ¹H NMR

(400 MHz, DMSO): 1.16 (3H, d, *J* = 4.6), 1.17 (3H, d, *J* = 4.4), 1.37 – 1.57 (3H, m),

15 1.61 – 1.70 (1H, m), 1.76 – 1.86 (1H, m), 1.94 – 2.03 (1H, m), 2.33 – 2.46 (2H, m),

2.94 – 3.03 (2H, m), 4.03 – 4.08 (1H, m), 4.15 – 4.31 (4H, m), 4.88 (1H, hept, *J* =

6.3), 5.42 (2H, s), 5.96 (1H, t, *J* = 5.8), 6.01 (1H, s), 7.31 – 7.34 (2H, m), 7.39 – 7.43

(2H, m), 7.53 (1H, d, *J* = 8.1), 7.73 – 7.74 (2H, m), 7.88 (2H, d, *J* = 7.5), 8.28 (1H, d,

J = 7.5). ¹³C NMR (101 MHz, DMSO): 21.45, 21.49, 25.91, 26.72, 29.39, 36.22,

20 38.77, 46.68, 51.48, 54.06, 65.67, 68.02, 120.10 (2C), 125.36 (2C), 127.09 (2C),

127.65 (2C), 140.72 (2C), 143.80, 143.91, 155.96, 158.87, 171.05, 172.29, 194.04. IR

(KBr): 3435 vs, br, 3348 vs, br, sh, 3068 m, 2979 m, 2936 m, 2871 w, 2105 s, 1723 s,

1678 vs, br, 1610 s, sh, 1540 s, br, 1478 s, 1466 m, sh, 1450 m, 1386 s, sh, 1376 s,

1252 s, 1220 m, sh, 1184 m, sh, 1146 m, 1105 s, 1052 m, 1032 m, 760 m, 741 m, 621

25 m, 427 s cm⁻¹. ESI MS: 615 ([M + Na]⁺). HR ESI MS: calcd for C₃₀H₃₆O₇N₆Na

615.25377; found 615.25383.

Isopropyl 2-(2-amino-5-ureidopentanamido)-6-diazo-5-oxohexanoate (54).

Compound **53** (1.84 g, 3.11 mmol), was dissolved in dry

DMF (24 mL). Piperidine (767 μL, 7.76 mmol, 2.5 equiv.)

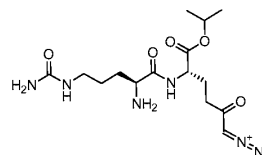
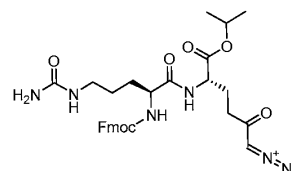
30 was added and the reaction mixture was stirred at room

temperature for 4 h. The organic solvent was evaporated in

vacuo. The residue was chromatographed on silica gel

(chloroform:methanol, 2:1) to afford the desired product **54** (874 mg, 76 %) as a white

amorphous solid. ¹H NMR (400 MHz, DMSO): 1.17 (3H, d, *J* = 4.4), 1.18 (3H, d, *J* =

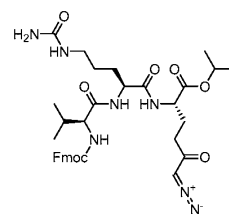


4.3), 1.27 – 1.47 (3H, m), 1.48 – 1.58 (1H, m), 1.76 – 1.85 (1H, m), 1.93 – 2.01 (1H, m), 2.31 – 2.43 (2H, m), 2.93 (2H, q, $J = 6.4$), 3.14 (1H, dd, $J = 7.3, 5.3$), 4.88 (1H, hept, $J = 6.3$), 5.36 (2H, s), 5.90 (1H, t, $J = 5.7$), 6.07 (1H, s), 8.13 (1H, d, $J = 7.7$).

^{13}C NMR (101 MHz, DMSO): 21.45, 21.50, 26.08, 26.48, 32.69, 36.25, 51.22, 54.21,

- 5 68.03, 158.72, 171.18, 175.56, 194.05. **IR** (CHCl_3): 3509 w, 3414 w, sh, 3446 w, 3357 m, br, sh, 3116 w, 2939 w, 2110s, 1731 s, 1664 vs, br, 1598 m, 1467 w, 1450 m, 1387 m, sh, 1377 s, 1349 m, 1183 w, 1145 m cm^{-1} . **Optical rotation**: $[\alpha]_D^{22} - 3.2^\circ$ (c 0.218, CH_2Cl_2). **ESI MS**: 371 ($[\text{M} + \text{H}]^+$). **HR ESI MS**: calcd for $\text{C}_{31}\text{H}_{40}\text{O}_{15}\text{N}_3\text{Na}$ 393.18569; found 393.18575.

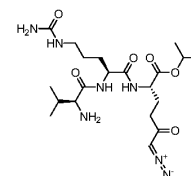
- 10 **Isopropyl 11-(4-diazo-3-oxobutyl)-1-(9H-fluoren-9-yl)-5-isopropyl-3,6,9-trioxo-8-(3-ureidopropyl)-2-oxa-4,7,10-triazadodecan-12-oate (55)**. Fmoc-Val-COOH (484 mg, 1.43 mmol, 1.1 equiv.) and TBTU (499 mg, 1.55 mmol, 1.2 equiv.) were solved in absolute DMF (10 mL) and diisopropylethyl amine (677 μL , 3.89 mmol, 3 equiv.) was
- 15 added. The reaction mixture was stirred at room temperature for 30 min. and then the solution of compound **54** (480 mg,



- 1.30 mmol) in absolute DMF (5 mL) were added by syringe. The reaction mixture was stirred for 3 h at room temperature under inert atmosphere. The organic solvent was evaporated *in vacuo*. The residue was chromatographed on silica gel
- 20 (chloroform:methanol, 15 : 1) to afford the desired product **55** (750 mg, 84 %) as a white amorphous solid. ^1H NMR (400 MHz, DMSO): 0.84 (3H, d, $J = 6.7$), 0.86 (3H, d, $J = 6.8$), 1.16 (3H, d, $J = 4.5$), 1.17 (3H, d, $J = 4.5$), 1.32 – 1.54 (3H, m), 1.60 – 1.68 (1H, m), 1.73 – 1.82 (1H, m), 1.91 – 2.04 (2H, m), 2.30 – 2.46 (2H, m), 2.90 – 3.02 (2H, m), 3.90 (1H, dd, $J = 9.1, 7.0$), 4.13 – 4.18 (1H, m), 4.20 – 4.34 (4H, m),
- 25 4.87 (1H, hept, $J = 6.2$), 5.41 (2H, s), 5.94 (1H, t, $J = 5.8$), 6.04 (1H, s), 7.30 – 7.34 (2H, m), 7.39 – 7.45 (3H, m), 7.72 – 7.76 (2H, m), 7.89 (2H, d, $J = 7.5$), 8.01 (1H, d, $J = 7.6$), 8.31 (1H, d, $J = 7.4$). ^{13}C NMR (101 MHz, DMSO): 18.26, 19.26, 21.48, 21.52, 25.99, 26.57, 29.54, 30.51, 36.09, 38.79, 46.71, 51.37, 52.15, 60.05, 65.68, 68.04, 120.15 (2C), 125.41 (2C), 127.12 (2C), 127.68 (2C), 140.72, 140.75, 143.79,
- 30 143.93, 156.12, 158.84, 171.03, 171.11, 171.76, 194.15. **IR** (KBr): 3415 m, vbr, sh, 3360 m, br, 3283 m, 3068 w, 2964 w, 2937 w, 2873 w, 2106 m, 1727 m, 1686 s, 1655 vs, sh, 1645 vs, 1540 s, br, 1478 w, 1465 m, 1451 m, 1386 m, sh, 1376 m, 1293 m, 1249 m, 1226 m, 1183 w, sh, 1146 m, 1106 m, 1033 w, 1009 vw, 760 w, 741 w, 621

vw, 427 vw cm^{-1} . **Optical rotation:** $[\alpha]_{\text{D}}^{22} - 19.3^\circ$ (c 0.114, DMSO). **ESI MS:** 714 ($[\text{M} + \text{Na}]^+$). **HR ESI MS:** calcd for $\text{C}_{35}\text{H}_{45}\text{N}_7\text{O}_8\text{Na}$ 714.32218; found 714.32218.

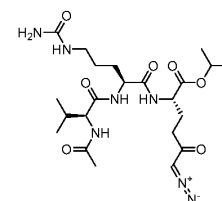
Isopropyl 2-(2-(2-amino-3-methylbutanamido)-5-ureidopentanamido)-6-diazo-5-oxohexanoate (56). Compound **55** (200 mg, 0.289 mmol), was dissolved in dry DMF (3 mL). Piperidine (71 μL 0.723 mmol, 2.5 equiv.) was added and the reaction mixture was stirred at room temperature for 4 h. The organic solvent was evaporated in vacuo. The residue was chromatographed on silica gel (chloroform:methanol, 2:1) to afford the desired product **56** (110 mg, 81 %) as a white



amorphous solid. **^1H NMR** (400 MHz, DMSO): 0.77 (3H, d, $J = 6.8$), 0.87 (3H, d, $J = 6.9$), 1.16 (3H, d, $J = 5.3$), 1.18 (3H, d, $J = 5.2$), 1.30 – 1.52 (3H, m), 1.57 – 1.68 (1H, m), 1.73 – 1.83 (1H, m), 1.86 – 2.01 (2H, m), 2.34 – 2.44 (2H, m), 2.89 – 3.00 (2H, m), 4.06 – 4.18 (2H, m), 4.29 – 4.37 (1H, m), 4.88 (1H, hept, $J = 6.3$), 5.37 (2H, s), 5.91 (1H, t, $J = 5.8$), 6.05 (1H, s), 8.04 (1H, d, $J = 7.8$), 8.34 (1H, d, $J = 7.4$). **^{13}C NMR** (101 MHz, DMSO): 17.49, 18.76, 21.46, 21.50, 25.86, 26.44, 29.61, 30.44, 36.08, 38.64, 51.45, 51.93, 57.96, 68.04, 158.99, 169.93, 170.95, 171.58, 194.03. **IR** (KBr): 3500 w, br, sh, 3338 m, vbr, 3116 w, 2984 s, 2965 s, 2936 m, 2874 m, 2109 s, 1731 s, 1653 vs, br, 1602 s, br, sh, 1552 s, br, 1517 s, 1466 m, 1452 m, 1387 s, sh, 1376 s, 1234 s, 1183 m, 1145 s, 1106 s cm^{-1} . **Optical rotation:** $[\alpha]_{\text{D}}^{22} - 13.0^\circ$ (c 0.270, DMSO). **ESI MS:** 470 ($[\text{M} + \text{H}]^+$). **HR ESI MS:** calcd for $\text{C}_{20}\text{H}_{36}\text{O}_6\text{N}_7$ 470.27216; found 470.27208.

Synthesis of isopropyl 2-(2-(2-acetamido-3-methylbutanamido)-5-ureidopentanamido)-6-diazo-5-oxohexanoate (57)

Isopropyl 2-(2-(2-acetamido-3-methylbutanamido)-5-ureidopentanamido)-6-diazo-5-oxohexanoate (57). Compound **56** (50 mg, 0.107 mmol), was dissolved in dry DMF (1 mL) and to this solution was added diisopropylethyl amine (95 μL , 0.543 mmol, 5.1 equiv.) was added dropwise followed by acetic anhydride (50 μL , 0.532 mmol, 5.0 equiv.). The reaction mixture was stirred at room temperature for 2 h. The organic solvent was evaporated *in vacuo*. The residue was chromatographed on silica gel (chloroform:methanol, 7:1) to afford the desired product **57** (50 mg, 92 %) as a yellow amorphous solid. **^1H NMR** (400 MHz, CDCl_3): 0.82 (3H, d, $J = 6.9$), 0.84 (3H, d, $J = 7.1$), 1.16 (3H, d, $J = 4.8$), 1.17 (3H, d, $J = 4.9$), 1.31 – 1.53 (3H, m), 1.60 – 1.68 (1H,



m), 1.73 – 1.82 (1H, m), 1.86 (3H, s), 1.89 – 2.00 (2H, m), 2.31 – 2.44 (2H, m), 2.93 – 2.97 (2H, m), 4.12 – 4.17 (2H, m), 4.21 – 4.26 (1H, m), 4.87 (1H, hept, $J = 6.3$), 5.39 (2H, s), 5.92 (1H, t, $J = 5.8$), 6.02 (1H, s), 7.86 (1H, d, $J = 8.7$), 7.97 (1H, d, $J = 7.6$), 8.20 (1H, d, $J = 7.5$). ^{13}C NMR (101 MHz, CDCl_3): 18.20, 19.21, 21.45, 21.49, 22.51, 25.95, 26.56, 29.36, 30.40, 36.10, 38.77, 51.33, 52.11, 57.67, 68.01, 158.80, 169.37, 170.97, 171.05, 171.70, 194.05. **Optical rotation:** $[\alpha]_{\text{D}}^{22} - 22.6^\circ$ (c 0.257, DMSO). **ESI MS:** 534 ($[\text{M} + \text{Na}]^+$). **HR ESI MS:** calcd for $\text{C}_{22}\text{H}_{37}\text{O}_7\text{N}_7\text{Na}$ 534.26467; found 513.26456.

Synthesis of (S)-6-diazo-5-oxo-2-((((4-(((2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)benzyl)oxy)carbonyl)amino)hexanoate (59)

2-(acetoxymethyl)-6-(4-(((6-diazo-1-ethoxy-1,5-dioxohexan-2-yl)carbamoyl)oxy)methyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (58). 4-[[[(4-Nitrophenoxy)carbonyl]oxy]methyl]phenyl- β -D-

glucopyranoside, 2,3,4,6-tetraacetate (prepared using a method analogous to the one reported in Angew.

Chem. Int. Ed. 2006, 45, 5345–5348, 800 mg, 1.3

mmol), was dissolved in dry DMF (6 mL) and a

solution of the compound **20** (330 mg, 1.66 mmol, 1.3 equiv.), in dry DMF (3 mL)

was added dropwise. To this reaction mixture was diisopropylethyl amine (0.91 mL, 5.2 mmol, 4 equiv.) was added dropwise. The reaction mixture was stirred at room temperature overnight. The organic solvent was evaporated *in vacuo*. The residue was chromatographed on silica gel (chloroform:acetone, 7:1) to effort the desired product **58** (596 mg, 68 %) as a yellow amorphous solid. ^1H NMR (400 MHz, CDCl_3): 1.25

(3H, m), 1.93 – 2.04 (1H, m), 2.02 (3H, s), 2.03 (3H, s), 2.04 (3H, s), 2.06 (3H, s), 2.15 – 2.23 (1H, m), 2.28 – 2.51 (2H, m), 3.85 (1H, ddd, $J = 10.0, 5.3, 2.5$), 4.13 – 4.20 (3H, m), 4.25 – 4.34 (2H, m), 5.03 – 5.07 (3H, m), 5.13 – 5.17 (1H, m), 5.21 – 5.31 (3H, m), 5.49 (1H, d, $J = 7.9$), 6.94 – 6.97 (2H, m), 7.27 – 7.31 (2H, m). ^{13}C NMR (101 MHz, CDCl_3): 14.24, 20.71, 20.72, 20.80, 21.82, 27.59, 36.50, 53.61,

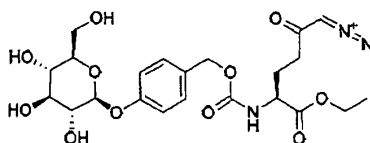
54.87, 61.80, 62.01, 66.56, 68.34, 71.23, 72.16, 72.78, 99.13, 117.05 (2C), 129.95 (2C), 131.38, 156.06, 156.86, 169.39, 169.50, 170.32, 170.66, 171.91, 193.55. **IR** (CHCl_3): 3429 w, 2110 s, 1757 vs, 1744 sh, vs, 1720 sh, s, 1641 m, 1613 m, 1592 w, 1512 s, 1377 m, 1368 s, 1178 m, 1070 sh, s, 651 w cm^{-1} . **Optical rotation:** $[\alpha]_{\text{D}}^{22} -$

3.3° (c 0.631, CH₂Cl₂). ESI MS: 680 ([M + H]⁺). HR ESI MS: calcd for

C₃₀H₃₈O₁₅N₃ 680.22974; found 680.22998.

Ethyl (S)-6-diazo-5-oxo-2-((((4-(((2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)benzyl)oxy)carbonyl)amino)

- 5 **hexanoate (59)**. Compound 58 (833 mg, 1.3 mmol), was dissolved in methanol (20 mL) and a hydrazine hydrate solution 50-60 % in water (417 µL, 7.36 mmol, 6.0 equiv.) was added dropwise. The reaction mixture was stirred at room temperature overnight. The organic solvent was
- 10 evaporated *in vacuo*. The residue was chromatographed on silica gel (chloroform - methanol 7:1) to effort the desired product 59 (240 mg, 37 %) as yellow amorphous solid. ¹H NMR (400 MHz, DMSO): 1.18 (3H, t, *J* = 7.1), 1.72 – 1.81 (1H, m), 1.91 – 2.00 (1H, m), 2.34 – 2.45 (2H, m), 3.12 – 3.19 (1H, m), 3.20 – 3.28 (2H, m), 3.29 – 3.34 (1H, m), 3.42 – 3.48 (1H, m),
- 15 3.66 – 3.71 (1H, m), 3.97 – 4.03 (1H, m), 4.05 – 4.12 (2H, m), 4.54 (1H, t, *J* = 5.8), 4.86 (1H, d, *J* = 7.3) 4.96 (2H, s), 5.01 (1H, d, *J* = 5.3), 5.08 (1H, d, *J* = 4.6), 5.30 (1H, d, *J* = 4.8), 6.05 (1H, s), 7.01 (2H, d, *J* = 8.6), 7.28 (2H, d, *J* = 8.6), 7.67 (1H, d, *J* = 7.8). ¹³C NMR (101 MHz, DMSO): 14.07, 25.82, 36.30, 53.27, 60.59, 60.70, 65.30, 69.72, 73.23, 76.63, 77.04, 100.28, 116.11 (2C), 129.49 (2C), 130.09, 156.16,
- 20 157.14, 172.06, 201.32. IR (KBr): 3413 m, 2979 w, 2935 w, 2108 m, 1718m, 1649 m, 1614 m, 1592 w, 1513 m, 1392 m, sh, 1383 m, 1346 m, 1233 s, 1179 m, sh, 1074 s, 1046 s, sh, 1018 m, 948 w, sh, 832 w, 511 w cm⁻¹. Optical rotation: [α]_D²² - 18.9° (c 0.254, DMSO). ESI MS: 534 ([M + Na]⁺). HR ESI MS: calcd for C₂₂H₂₉O₁₁N₃Na 534.16943; found 534.16951.



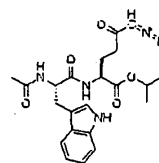
- 25 **Synthesis of isopropyl 2-(2-acetamido-3-(1H-indol-3-yl)propanamido)-6-diazo-5-oxohexanoate (60)**

Isopropyl 2-(2-acetamido-3-(1H-indol-3-yl)propanamido)-6-diazo-5-

oxohexanoate (60). Compound 38 (425 mg, 1.06 mmol, 1 eq.) was dissolved in dry

DMF (8 mL). Pyridine (168 mg, 171 µL, 2.13 mmol, 2 eq.),

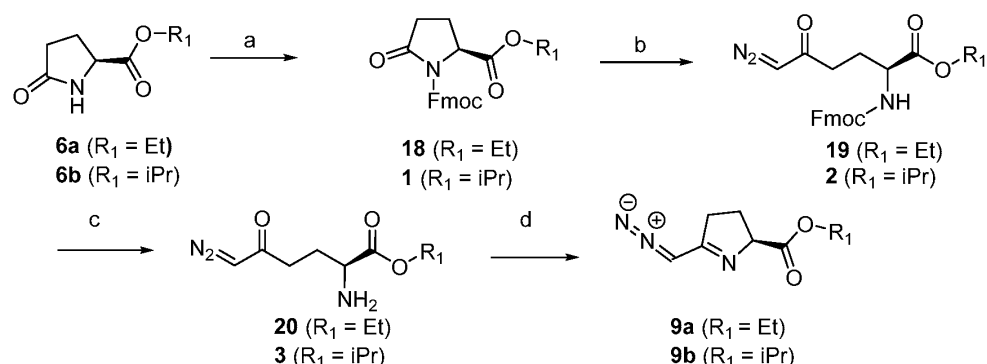
- 30 followed by acethanhydride (130 mg, 121 µL, 1.28 mmol, 1.2 eq.) were added by syringe. The reaction mixture was stirred for 2 h at rt under inert atmosphere. DMF was evaporated. The crude product was purified by column LC (CHCl₃:MeOH, 20:1) to give 423 mg of product 60 as a yellow amorphous solid (90 % yield). ¹H



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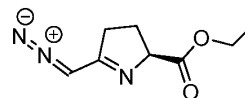
NMR (400 MHz, CDCl₃): 1.20 (3H, d, $J = 6.3$), 1.24 (3H, d, $J = 6.3$), 1.81 – 1.94 (1H, m), 1.98 (3H, s), 2.04 – 2.33 (3H, m), 3.17 (1H, dd, $J = 14.6, 7.2$), 3.33 (1H, dd, $J = 14.7, 5.4$), 4.37 (1H, td, $J = 7.7, 4.4$), 4.75 (1H, td, $J = 7.4, 5.4$), 4.95 (1H, hept, $J = 6.3$), 5.16 (1H, bs), 6.22 (d, $J = 7.6$), 6.64 (1H, d, $J = 7.3$), 7.09 – 7.14 (2H, m), 7.18 (1H, ddd, $J = 8.2, 7.0, 1.3$), 7.32 – 7.38 (1H, m), 7.66 (1H, dd, $J = 7.9, 1.1$), 8.31 (1H, bs). **¹³C NMR** (101 MHz, CDCl₃): 21.77, 21.82, 23.38, 26.97, 28.29, 36.25, 52.28, 54.07, 54.95, 69.54, 110.38, 111.42, 118.78, 119.79, 122.27, 123.57, 127.74, 136.35, 170.25, 170.85, 171.58, 194.04. **Optical rotation**: $[\alpha]_D^{22} - 11.6^\circ$ (c 0.284, CHCl₃). **IR** (CHCl₃): 3478 m, 3417 m, 3329 w, vbr, 3117 w, 2986 m, 2934 w, 2874 vw, 2855 w, 2110 s, 1732 s, 1660 vs, br, 1635 s, sh, 1600 w, sh, 1554 m, br, sh, 1505 s, br, 1467 w, 1457 m, 1385 s, sh, 1377 w, vbr, 1350 m, 1183 m, 1146 m, 1105 s, 1093 m, sh, 1012 w. **ESI MS**: 464 ($[M + Na]^+$). **HR ESI MS**: calcd for C₂₂H₂₇O₅N₅Na 464.19044; found 464.19050.

15 **Scheme 9**



Reagents and conditions: (a) LiHMDS, Fmoc-Cl, THF, -78°C, 87% for **7a**, 92% for **7b**; (b) n-BuLi, TMS diazomethane, THF, -116 to -78 °C, 80% for **8a**, 85% for **8b**; (c) Piperidine, DCM, rt, 66% for **2a** and 66% for **2b**; (d) CH₃CN, 60 °C, 50% for **9a**; CDCl₃, rt, overnight, 41% for **9b**.

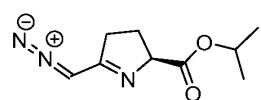
Ethyl 5-(diazomethyl)-3,4-dihydro-2H-pyrrole-2-carboxylate (9a) Referring to scheme 9, compound **20** (117 mg, 0.588 mmol, 1 eq.) was dissolved in CH₃CN (1 mL). The reaction mixture was heated for 2 h at 60 °C under inert atmosphere. The CH₃CN was evaporated and the crude product was purified by HPLC (CH₃CN/H₂O, HCOOH) affording a light orange oil (53 mg) in 50% yield. **¹H NMR** (CDCl₃): 1.27 (3H, t, $J = 7.1$), 2.83 – 2.93 (1H, m), 2.93 – 3.07 (2H, m), 3.09 – 3.20 (1H, m), 4.24 (2H, dq, $J =$



7.1, 2.4), 5.17 (1H, dd, $J = 9.2, 3.5$), 7.43 (1H, s); ^{13}C NMR (101 MHz, CDCl_3): 14.11, 19.98, 34.19, 59.06, 62.53, 126.82, 142.77, 168.70; **IR** (CHCl_3): 2942 w, 2910 w, 2875 vw, 2103 vw, 1747 vs, 1475 w, 1676 m, 1605 vw, 1552 w, 1462 w, 1446 w, 1396 w, 1377 m, 1202 vs, 1177 m, 1116 w, 1095 m, cm^{-1} ; **ESI MS**: 182 ($[\text{M} + \text{H}]^+$);

5 **HR ESI MS**: calcd for $\text{C}_8\text{H}_{12}\text{O}_2\text{N}_3$ 182.0930; found 182.0931.

Isopropyl 5-(diazomethyl)-3,4-dihydro-2H-pyrrole-2-carboxylate (9b). Referring



to scheme 9, compound **3** (100 mg, 0.469 mmol, 1 eq.) was dissolved in CDCl_3 (2 mL). The reaction mixture was stirred at rt overnight. The CDCl_3 was evaporated and the

10 crude product was purified by HPLC ($\text{CH}_3\text{CN}/\text{H}_2\text{O}$, HCOOH) and a light orange oil (41 mg) was obtained in 45% yield. ^1H NMR (CDCl_3): 1.25 (3H, d, $J = 6.1$), 1.27 (3H, d, $J = 6.1$), 2.81 – 2.95 (2H, m), 2.96 – 3.06 (1H, m), 3.08 – 3.18 (1H, m), 5.09 (1H, hept, $J = 6.1$), 5.13 (1H, dd, $J = 9.1, 3.4$), 7.43 (1H, s); ^{13}C NMR (101 MHz, CDCl_3): 20.00, 21.69 (2C), 21.75, 34.24, 59.20, 70.48, 126.84, 142.71, 168.25;

15 **Optical rotation**: $[\alpha]_D^{22} - 31.8^\circ$ (c 0.110, CHCl_3); **IR** (CHCl_3): 2104 w, 1741 vs, 1675 w, 1644 w, 1551 w, 1465 m, 1388 m, sh, 1376 m, 1209 s, 1182 m, 1147 m, 1106 s, cm^{-1} ; **ESI MS**: 196 ($[\text{M} + \text{H}]^+$); **HR ESI MS**: calcd for $\text{C}_9\text{H}_{14}\text{O}_2\text{N}_3$ 196.10805; found 196.10808.

20

EXAMPLE 5

Evaluation of DON prodrugs

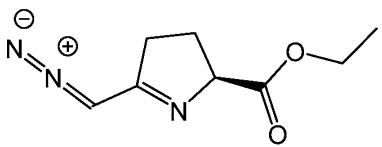
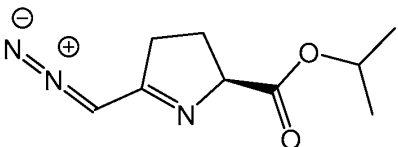
Overview. The presently disclosed subject matter demonstrates profound efficacy of DON in murine model of GBM, although overt toxicities were observed. In attempt to increase DON's therapeutic index, several DON prodrugs were

25 systematically synthesized. The initial strategy involved masking DON's carboxylic acid with simple alkyl esters, such as ethyl ester **20** and isopropyl ester **3** (FIG. 20). However, **3** and **20** exhibited chemical instability cyclizing to form a unique diazo-imine. Given this instability, both the primary amine and the carboxylate of DON were next masked with prodrug moieties. Three types of amine promoieties were

30 used, including (oxodioxolenyl)methyl carbamate esters (**13** and **36**), dipeptides (**9** and **25**), and pivaloyl-oxy-methyl (POM)-based esters (**14**, **32** and **42**). The dual promoiety-containing prodrugs resulted in sufficient chemical stability permitting further evaluation in *in vitro* metabolic stability assays. While all of the prodrugs exhibited rapid metabolism in mouse plasma, some provided excellent plasma

stability in monkeys and humans. When evaluated *in vivo*, the most stable DON prodrug (**5c**, methyl-POM-DON-isopropyl-ester) achieved 10-fold enhanced brain: plasma ratio versus DON in monkeys, thus providing a possible clinical path to DON utilization in GBM patients.

- 5 *Chemistry.* Scheme 9 outlines the synthesis and characterization of the ester based prodrugs **20** and **3** of DON and their subsequent cyclization to novel diazo-imines **9a-b**. The pyroglutamate esters **6a-b** (D'Andrea, et al., 2008) were FMOC-protected to afford compounds **18** and **1**. Formation of the diazoketones **19** and **2** was accomplished using TMS diazomethane in good yield. Rapid deprotection with
- 10 piperidine afforded the ester based prodrugs **20** and **3**. Unexpectedly slow cyclization was observed even under mild conditions (e.g. stirring in chloroform at room temperature) affording the novel diazo-imines **9a** and **9b**. It is believed that this is the first example of this functional group being described in the chemical literature. Attempts to avoid the cyclization of **20** and **3** by salt formation to protonate the amine
- 15 resulted in instability of the diazo group. Furthermore, unlike most imines, the 5-member cyclic diazo-imines **9a-b** were found to be stable, even at acidic pH, and did not convert back to DON esters (results not shown).

Table 3. Novel Diazo-imines	
9a	
9b	

- 20 Given their sufficient chemical stability, compounds **20** and **3** were utilized as synthetic intermediates for preparation of dual promoiety prodrugs (Schemes 8 and 2).

The (oxodioxolenyl) methyl carbamate adducts **36** and **13** were synthesized as outlined in Schemes 8 and 2. The 4-(hydroxymethyl)-5-methyl-1,3-dioxol-2-one was reacted with *S*-ethyl carbonochloridothionate, (Keicher, et al., 2009) followed by *N*-hydroxysuccinimide to provide **12**. Reaction of **20** and **3** with **12** afforded the (oxodioxolenyl) methyl carbamate esters **36** and **13** in excellent yields.

Schemes 1, 4, and 7 outline the synthesis of DON dipeptide esters. DON esters **20** and **3** were coupled with Fmoc-L-leucine or Fmoc-L-tryptophan using HBTU in high yield to form the protected dipeptides **33** and **37** (scheme 7). Deprotection with diethyl amine or piperidine afforded the desired leucine-DON **25** (scheme 4) and **9** (scheme 1) and tryptophan-DON prodrugs **34** and **38** (scheme 7).

As shown in Scheme 6, the POM derivative **42** was synthesized from **20** using the POM-*N*-hydroxysuccinimate ester (Gallop, et al., 2008) in 40% yield. Introduction of methyl group in the POM ester led to the formation of methyl-POM derivatives **32** (scheme 6) and **14** (scheme 2), with an added chiral center. Both **32** and **14** were synthesized from **20** and **3** using 1-(((2,5-dioxopyrrolidin-1-yl)oxy)carbonyl)oxyethyl pivalate (Gallop, et al., 2008). **32** was obtained as a mixture of two diastereomers (1:1 ratio) in 68% yield. **14** was also obtained diastereomeric mixture (1:1) ratio, but was further purified into its corresponding diastereomers 1 and 2, of which diastereomer **1** was used for subsequent biological testing as described below.

Results and discussion.

DON showed robust inhibition of glutamine metabolism and antitumor efficacy in a murine GBM model. Despite several lines of evidence indicating the potential therapeutic efficacy of targeting glutamine metabolism in GBM, the effect of DON on GBM tumor growth has not yet been reported *in vivo*. Using the U87 flank xenograft mouse model of GBM, (Eshleman, et al., 2002) it has been first confirmed that systemic administration of DON (0.8 mg/kg, i.p) inhibited glutamine metabolism as reflected by an accumulation of endogenous glutamine in the tumor (FIG. 21A; $p < 0.05$) similar to other model systems. (Willis, et al., 1977; Windmueller, et al., 1974) Then its antitumor efficacy was evaluated, and observed that DON not only halted tumor growth, but also effectively induced tumor regression. Specifically, vehicle-treated mice displayed significant tumor growth over the course of the experiment, while DON-treated mice (0.8 mg/kg, i.p, q.d.) exhibited >50% reduction in tumor volume (FIG. 21B; main effect of time [$F(3,48) = 6.049$, $p = 0.0014$]; treatment

[F(1,16) = 33.42, $p < 0.0001$]; interaction [F(3,48) = 21.70, $p < 0.0001$]). Although DON exhibited excellent anti-tumor efficacy, all mice receiving DON displayed significant signs of toxicity including weight loss ($12 \pm 4.1\%$), hunching, ptosis, and lethargy. These findings are consistent with other reports of DON's efficacy and toxicity both *in vitro* and *in vivo*. (Fogal, et al., 2015; Cervantes-Madrid, et al., 2015; Potter, et al., 2015)

Some simple DON alkyl ester prodrugs found to be unstable. Masking both DON's carboxylate and amine functionalities enhanced the stability of certain prodrugs. A prodrug strategy is often employed to enhance tissue penetration and change the pharmacokinetic parameters of effective drugs. Indeed, prodrug strategies are common in drug development as 5–7% of the approved worldwide drugs are prodrugs (Rautio, et al., 2008). The initial prodrug strategy for DON involved masking the carboxylic acid with simple alkyl esters such as ethyl **20** and isopropyl **3**. The synthesis of these two derivatives was straightforward affording compounds **20** and **3** in good yield. It is surprising that these simple DON alkyl esters had not previously been reported in the chemical literature, given that DON chemistry and utility has been described by numerous groups for over 60 years (Magill, et al., 1957; Dion, et al., 1956; Magill, et al., 1956; Coffey, et al., 1956). One potential reason is that it has been discovered that **20** and **3** were unstable, slowing cyclizing to form unique diazo-imines **9a** and **9b**. These two unique derivatives were found to be chemically stable even at acidic pH, precluding their use as DON prodrugs.

Given the instability of certain simple ester prodrugs, both the primary amine and the carboxylate of DON were masked with prodrug moieties. This dual promoiety strategy was rationalized to eliminate the potential for cyclization and potentially further improve the lipophilicity. Three amine promoieties including (oxodioxolonyl)methyl carbamate esters were used (FIG. 20, compounds **36**, **13**), dipeptides (**25** and **38**), and pivaloyl-oxy-methyl (POM)-based esters (**42**, **32**, **14**). These promoieties were chosen because they target distinct metabolic enzymes including paraoxonase, aminopeptidases, and carboxylesterases, respectively. To impart further metabolic stability of the POM derivative (Table 3, **42**), corresponding methyl-POM analogs were prepared (**32**, **14**). All dual promoiety-containing prodrugs exhibited sufficient chemical stability to permit further evaluation.

All DON prodrugs were rapidly metabolized in mouse plasma, however **32** and **14** found to be stable in human and monkey plasma. Table 3 outlines the plasma

stability of DON prodrugs **36**, **13**, **25**, **9**, **34**, **38**, **42**, **32** and **14**. All prodrugs were completely metabolized in mouse plasma within the 60 min incubation time.

However in monkey and human plasma, the prodrugs **32** and **14**, with methyl-POM on the amine and ethyl or isopropyl ester on the carboxylate respectively,

- 5 demonstrated moderate/high stability with 60-75% of the prodrug remaining in monkey plasma, and 80-90% remaining in human plasma within the 60 min incubation time. Given **14** had the best stability profile in human plasma, it was selected for further evaluation in pharmacokinetic studies and compared to DON for its ability to penetrate the brain and liberate DON.

10

Table 4			
Compound #	PLASMA STABILITY		
	<i>Mouse</i>	<i>Monkey</i>	<i>Human</i>
36	0	0	0
13	0	0	0
25	0	1	1
9	0	1	1
34	0	4	12
38	0	10	30
42	0	0	9
32	0	75	88
14	0	61	91

Lead prodrug **14** enhanced brain delivery of DON in monkeys but not in mice.

- As expected from a DON prodrug which is completely metabolized in mouse plasma, it has been found that oral administration of DON (**1**) (0.8 mg/kg) and **14** (0.8 mg/kg equivalent) exhibited similar DON plasma (FIG. 22A) and brain (FIG. 22B) concentration profiles when dosed in mice. The AUC_{0-t} of DON following administration of DON and **14** in plasma were 1.25 nmol*h/mL and 1.22 nmol*h/mL respectively, suggesting rapid and complete liberation of DON from **14** *in vivo*. Similarly in the mouse brain, the AUC_{0-t} of DON following DON or **14** administration was 0.57 nmol*h/g and 0.69 nmol*h/g, respectively, with the brain/plasma approximately 0.46 from DON vs 0.56 from prodrug **14**. These pharmacokinetic
- 15
- 20

results corroborated the *in vitro* metabolism studies suggesting **14** was completely converted to DON in mouse plasma.

Following the mouse studies, the pharmacokinetics of DON and **14a** were evaluated in monkeys, as monkeys better mimicked the human plasma stability profile. In pigtail macaques, i.v. administration of DON and **14** (1.6 mg/kg DON equivalent) demonstrated significantly different DON plasma profiles FIG. 23A). DON administration provided high plasma exposures with AUC_{0-t} of 42.7 nmol*h/mL. In contrast, **14** administration delivered about 7 fold lower plasma exposure of DON with AUC_{0-t} of 5.71 nmol*h/mL. The opposite observation was seen in the CSF where enhanced DON levels were observed after **14** administration. In the CSF at 30 min post dose, DON administration resulted in 0.33 nmol/g DON while **14** delivered 1.43 nmol/g DON. When comparing plasma to CSF ratio at 30 min, **14** demonstrated 10-fold enhancement of DON CSF delivery versus DON (FIG. 23B).

The glutamine antagonist 6-diazo-5-oxo-L-norleucine (DON, **1**) has shown robust anti-cancer efficacy in preclinical and clinical studies, but its development was halted due to marked systemic toxicities. Herein it has been demonstrated that DON inhibits glutamine metabolism and provides antitumor efficacy in a murine model of glioblastoma, although toxicity was observed. To enhance DON's therapeutic index, a prodrug strategy was used to increase its brain delivery and limit systemic exposure. Unexpectedly, simple alkyl ester-based prodrugs were ineffective due to chemical instability cyclizing to form a unique diazo-imine. However, masking both DON's amine and carboxylate functionalities imparted sufficient chemical stability for biological testing. While these dual moiety prodrugs exhibited rapid metabolism in mouse plasma, several provided excellent stability in monkey and human plasma. The most stable compound (**14**, methyl-POM-DON-isopropyl-ester) is highly stable in plasma from monkey, human, pigs and dog but is rapidly metabolized in mice. When evaluated in monkeys at an equimolar doses, **14** delivered lower levels of DON to plasma but higher levels in CSF it achieved 10-fold enhanced brain:plasma ratio versus DON. This strategy may provide a path to DON utilization in GBM patients.

REFERENCES

All publications, patent applications, patents, and other references mentioned in the specification are indicative of the level of those skilled in the art to which the presently disclosed subject matter pertains. All publications, patent applications, patents, and other references are herein incorporated by reference to the same extent as if each individual publication, patent application, patent, and other reference was specifically and individually indicated to be incorporated by reference. It will be understood that, although a number of patent applications, patents, and other references are referred to herein, such reference does not constitute an admission that any of these documents forms part of the common general knowledge in the art. In case of a conflict between the specification and any of the incorporated references, the specification (including any amendments thereof, which may be based on an incorporated reference), shall control. Standard art-accepted meanings of terms are used herein unless indicated otherwise. Standard abbreviations for various terms are used herein.

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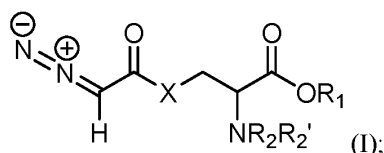
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20 Although the foregoing subject matter has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be understood by those skilled in the art that certain changes and modifications can be practiced within the scope of the appended claims.

THAT WHICH IS CLAIMED:

1. A prodrug of a glutamine analog, or a pharmaceutically acceptable salt or ester thereof, the prodrug having a structure of formula (I):



wherein:

X is selected from the group consisting of a bond, $-O-$, and $-(CH_2)_n-$, wherein n is an integer selected from the group consisting of 1, 2, 3, 4, 5, 6, 7, and 8;

R₁ is selected from the group consisting of H and a first prodrug-forming moiety capable of forming a salt or an ester; and

R₂ is H or a second prodrug-forming moiety capable of forming an amide linkage, a carbamate linkage, a phosphoramidate linkage or a phosphorodiamidate linkage with the nitrogen adjacent to R₂;

R₂' is selected from the group consisting of H, C₁-C₆ alkyl, substituted C₁-C₆ alkyl, or

R₂ and R₂' together form a ring structure comprising $-C(=O)-G-C(=O)-$, wherein G is selected from the group consisting of C₁-C₈ alkylene, C₁-C₈ heteroalkylene, C₅-C₈ cycloalkylene, C₆-C₁₂ arylene, C₅-C₁₄ heteroarylene, bivalent C₄-C₁₀ heterocycle, each of which can be optionally substituted; or

R₁ and R₂' together form a 4-to 6-membered heterocyclic ring comprising the oxygen atom adjacent to R₁ and the nitrogen atom adjacent to R₂';

provided that the compound has at least one prodrug-forming moiety selected from the group consisting of the first and the second prodrug-forming moieties.

2. The prodrug of claim 1, wherein X is $-CH_2-$.

3. The prodrug of claim 1, wherein X is $-O-$.

4. The prodrug of claim 1, wherein the glutamine analog is a glutamine antagonist selected from the group consisting of 6-diazo-5-oxo-norleucine (DON), 5-diazo-4-oxo-L-norvaline (L-DONV), and aza-serine.

5. The prodrug of claim 1, wherein R_1 comprises a residue PRO_1 of the prodrug-forming moiety, which, together with:
- a basic moiety and a terminal hydroxyl group forms a salt;
 - an alkyl group and the oxygen of an adjoining hydroxyl group forms an ester; or
 - an alkyl group and the nitrogen atom adjoining R_2' forms an azlactone or an oxazolidone.
6. The prodrug of claim 1, wherein R_1 is selected from the group consisting of H, alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkenyl, substituted cycloalkenyl, tri(hydrocarbyl)ammonium, and tetra(hydrocarbyl)ammonium.
7. The prodrug of claim 6, wherein R_1 is selected from the group consisting of a C_{1-6} straight-chain alkyl, a substituted C_{1-6} straight-chain alkyl, a C_{1-6} branched alkyl, a substituted C_{1-6} branched alkyl, tri(C_1 - C_8 -alkyl)ammonium, tetra(C_1 - C_8 -alkyl)ammonium, triphenylammonium, tri(hydroxy- C_1 - C_8 -alkyl)ammonium, and tetra(hydroxy- C_1 - C_8 -alkyl)ammonium.
8. The prodrug of claim 1, wherein R_1 is selected from the group consisting of methyl, ethyl, isopropyl, cyclopentyl, cyclohexyl, trimethylammonium, triethylammonium, tri(hydroxyethyl)ammonium, tripropylammonium, and tri(hydroxypropyl)ammonium.
9. The prodrug of claim 1, wherein R_2 comprises a residue PRO_2 of the second prodrug-forming moiety which comprises a carbonyl, an oxy carbonyl, or a phosphonyl group, wherein the carbonyl, the oxy carbonyl, or the phosphonyl group is bound to the nitrogen of the adjoining NR_2' to form an amide, a carbamate, phosphoramidate, or phosphorodiamidate linkage.
10. The prodrug of claim 9, wherein PRO_2 comprises a moiety selected from the group consisting of an amino acid, an N-substituted amino acid, a peptide, a substituted peptide, a monocyclic ring, a substituted monocyclic ring, a bicyclic ring,

a substituted bicyclic ring, a purine nucleoside, a substituted purine nucleoside, a pyrimidine nucleoside, and a substituted pyrimidine nucleoside.

11. The prodrug of claim 1, wherein R_2 is selected from the group consisting of H, alkyl, $-C(=O)-Ar$, $-C(=O)-Y-(CR_3R_4)_m-Ar$, $-C(=O)-Y-(CR_3R_4)_m-NR_5R_6$, $-P(=O)(OR_7)_n(NHR_9)_o$, $-C(=O)-Y-(CR_3R_4)_m-Ar-O-C(=O)-R_8$, $-C(=O)-Y-(CR_3R_4)_m-Ar-O-R_8$, $-C(=O)-O-(CR_3R_4)_m-O-C(=O)-R_{10}$, $-C(=O)-O-R_9$, $-C(=O)-Y-(CR_3R_4)_m-Ar-O-C(=O)-Ar$, and $-C(=O)-Y-(CR_3R_4)_m-Ar-NR_5R_6$;

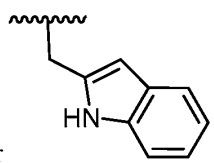
wherein:

Y is $-O-$ or a bond;

m is an integer selected from the group consisting of 0, 1, 2, 3, 4, 5, 6, 7, and 8;

each n and o is an integer from 0 to 2 provided that the sum of n and o is 2;

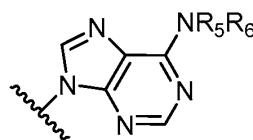
each R_3 and R_4 is independently H, C_1-C_6 alkyl or substituted C_1-C_6 alkyl, aryl



or substituted aryl, $-(CR_3R_4)_m-NR_5R_6$, or

each R_5 and R_6 is independently H, alkyl, $-C(=O)-(CR_3R_4)_m$, $-C(=O)-(NR_5R_6)$, or $-C(=O)-(CR_3R_4)_m-NR_5R_6$;

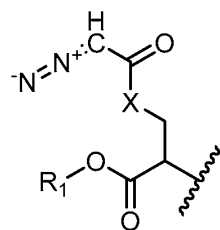
each R_7 is independently selected from the group consisting of H, alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, heterocyclyl, substituted heterocyclyl, alkenyl, substituted alkenyl, cycloalkenyl, substituted cycloalkenyl, $-(CR_3R_4)_m-Z$, $-(CR_3R_4)_m-Q-Z$ wherein Q is a monosaccharide, aryl, substituted aryl,



heteroaryl, substituted heteroaryl, and wherein Z is

or wherein R_7 together with the oxygen atom to which it is attached forms a purine or pyrimidine nucleoside;

each R_9 is independently selected from the group consisting of H, alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, heterocyclyl, substituted heterocyclyl, alkenyl, substituted alkenyl, cycloalkenyl, substituted cycloalkenyl, $-(CR_3R_4)_m-Z$, aryl, substituted aryl, heteroaryl, substituted heteroaryl, and



, wherein R_1 and X are as defined in claim 1, provided

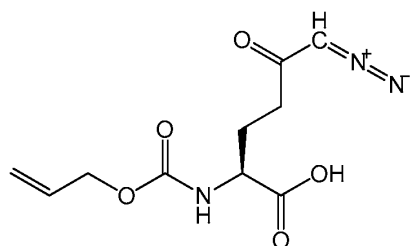
that R_1 is not H,

each R_8 is independently alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, monosaccharide, acylated monosaccharide, aryl, substituted aryl, heteroaryl, substituted heteroaryl;

each R_{10} is independently alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, monosaccharide, acylated monosaccharide, aryl, substituted aryl, heteroaryl, substituted heteroaryl,; and

Ar is aryl, substituted aryl, heteroaryl, or substituted heteroaryl.

12. The prodrug of claim 11, wherein the compound of formula (I) is



13. The prodrug of claim 11, wherein R_2 is $-C(=O)-Y-(CR_3R_4)_m-NR_5R_6$; wherein:

(i) Y is a bond;

m is 1;

R_5 and R_6 are each H; or

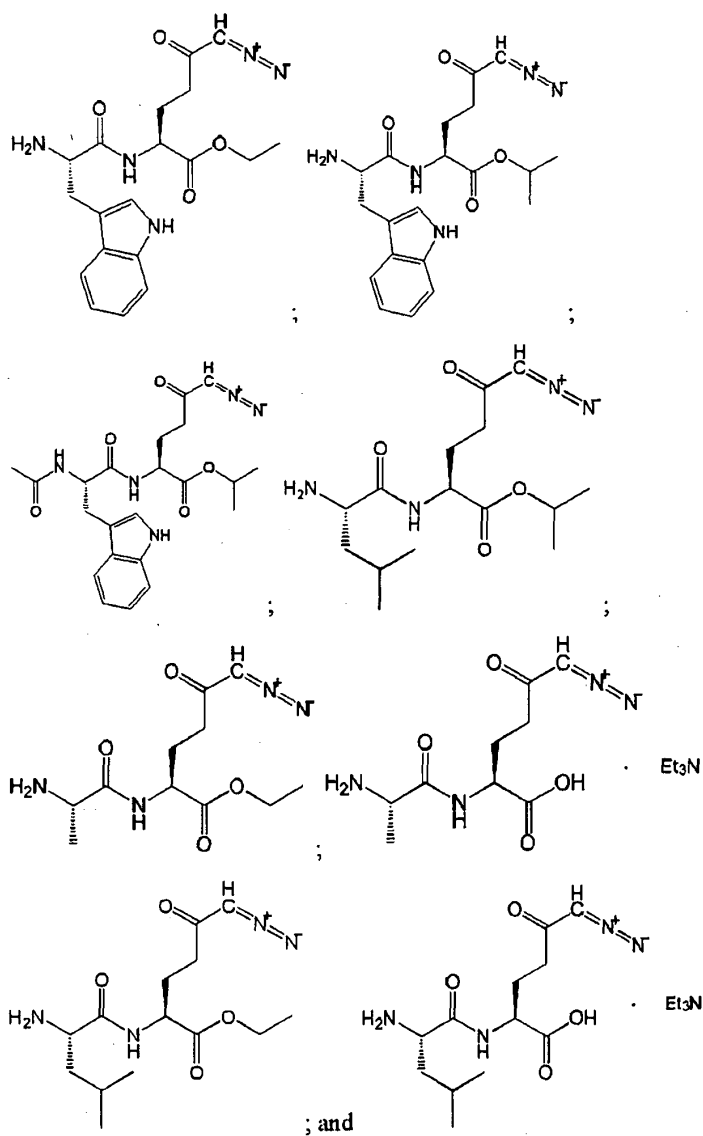
(ii) Y is a bond;

m is 1;

R_5 is H;

R_6 is $-C(=O)-(CR_3R_4)_m$.

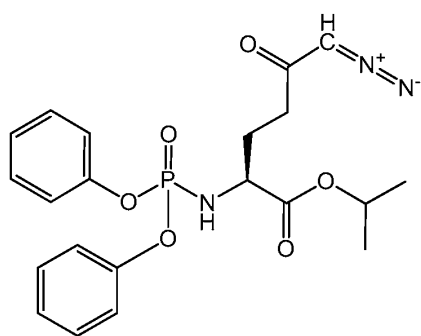
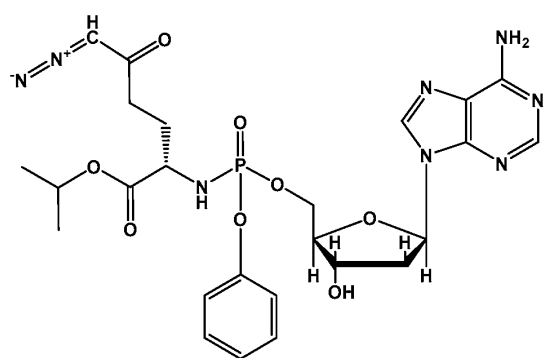
14. The prodrug of claim 13, wherein the compound of formula (I) is selected from the group consisting of:

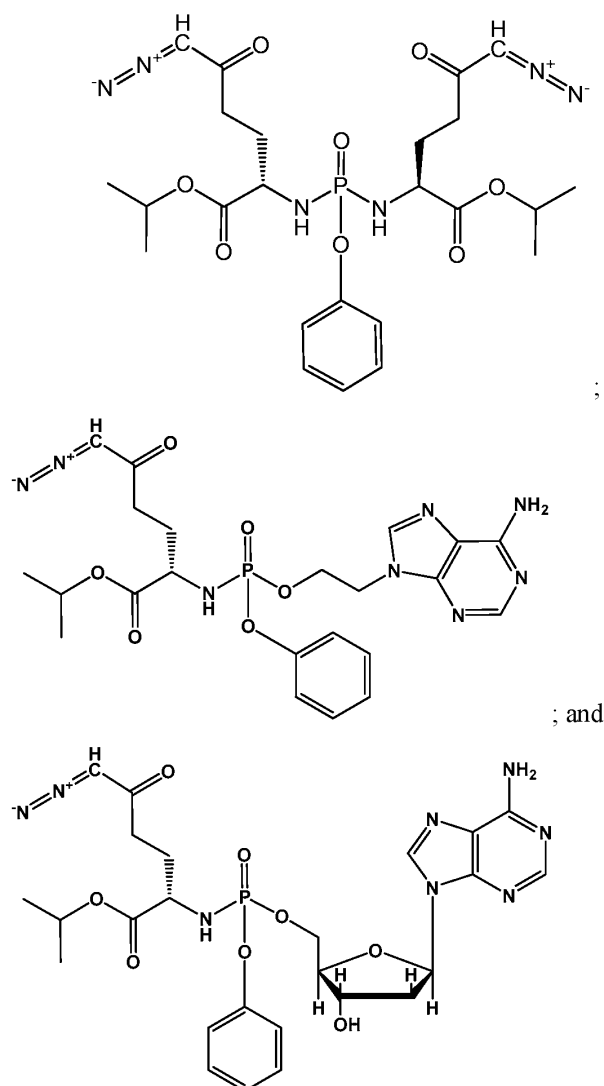


15. The prodrug of claim 11, wherein R_2 is $-P(=O)(OR_7)_n(NHR_9)_o$; wherein:

- (i) n is 2 and o is 0;
- (ii) n is 1 and o is 1; or
- (iii) n is 0 and o is 2.

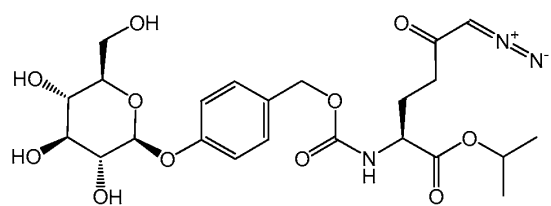
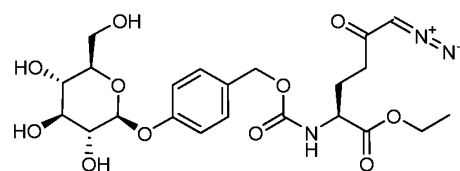
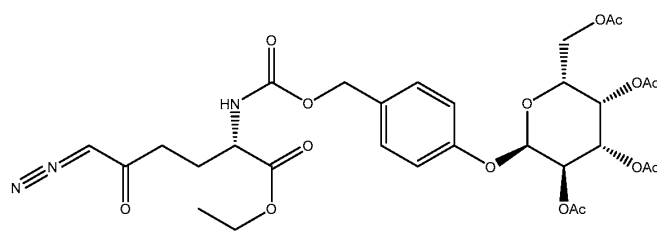
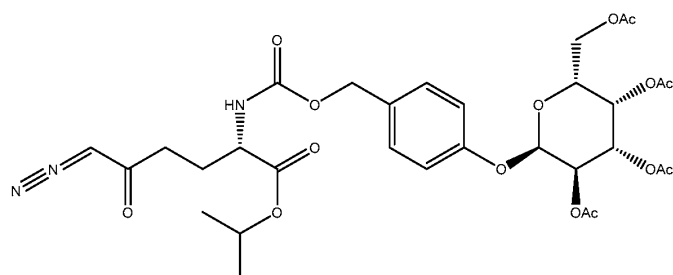
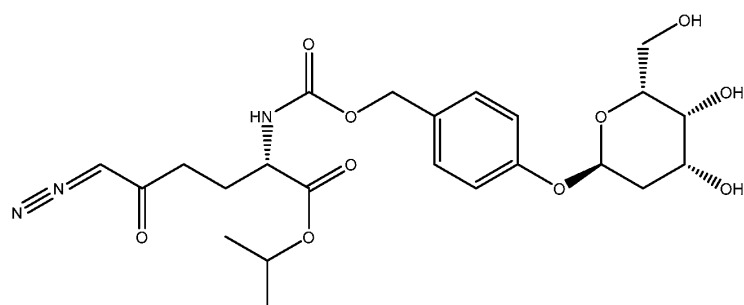
16. The prodrug of claim 15, wherein the compound of formula (I) is selected from the group consisting of:

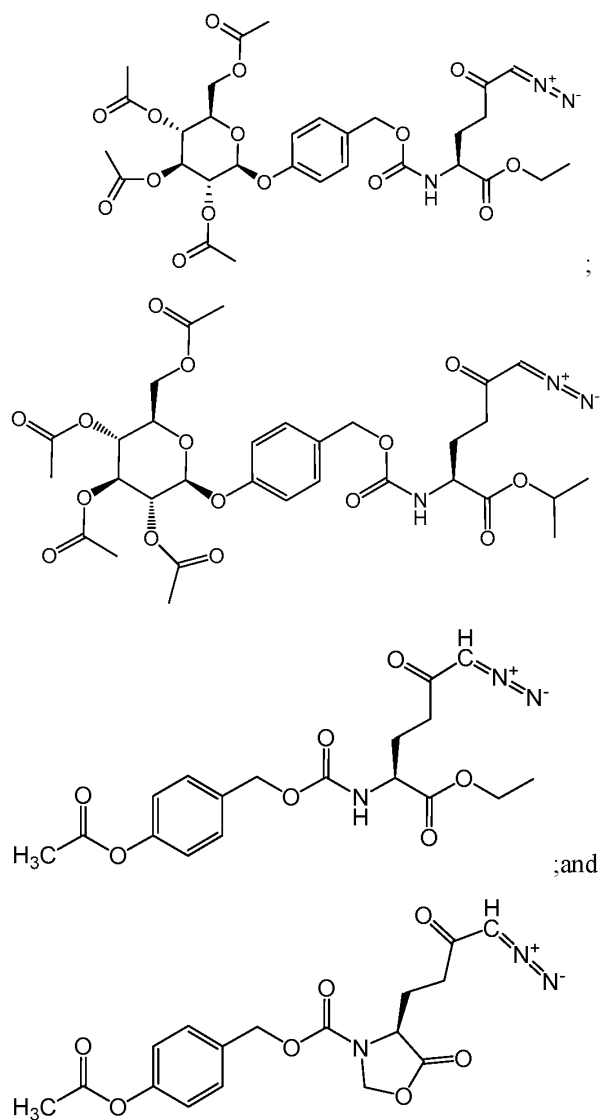




17. The prodrug of claim 11, wherein: R_2 is $-C(=O)-Y-(CR_3R_4)_m-Ar-O-C(=O)-R_8$ or $-C(=O)-Y-(CR_3R_4)_m-Ar-O-R_8$; and
 Y is $-O-$;
 m is 0; and
 Ar is benzyl.

18. The prodrug of claim 17, wherein the compound of formula (I) is selected from the group consisting of:





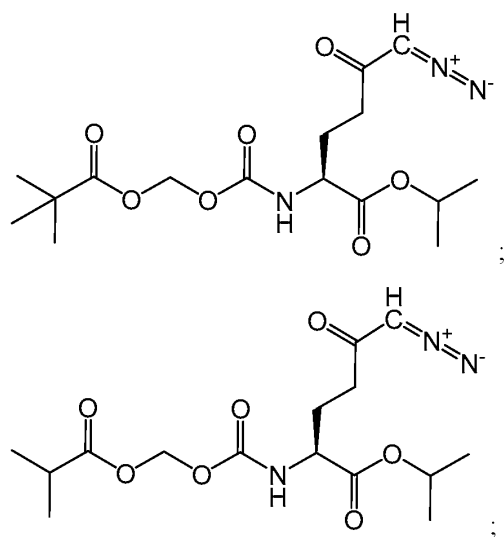
19. The prodrug of claim 11, wherein R_2 is $-C(=O)-O-(CR_3R_4)_m-O-C(=O)-R_{10}$;

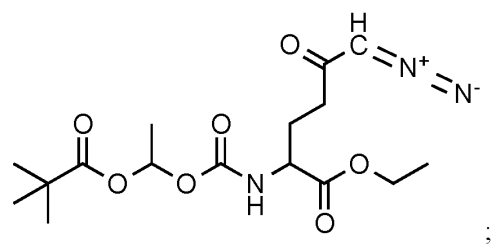
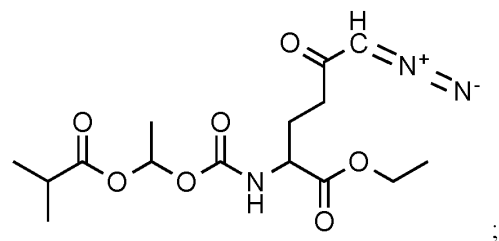
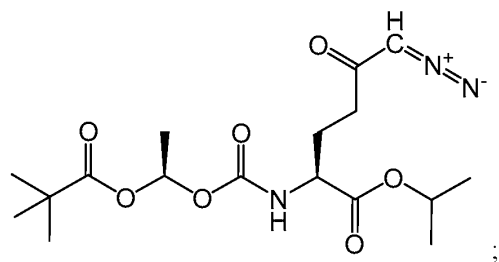
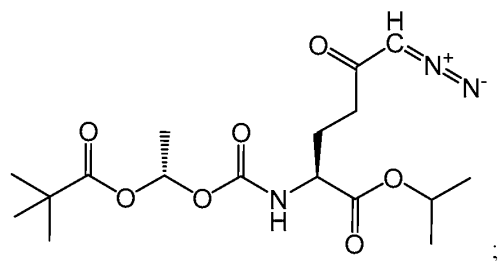
wherein:

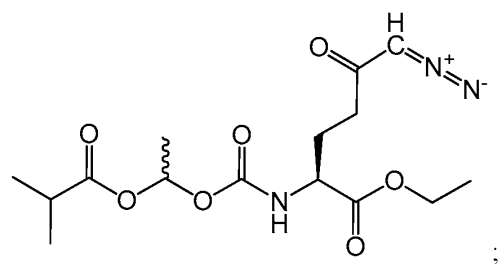
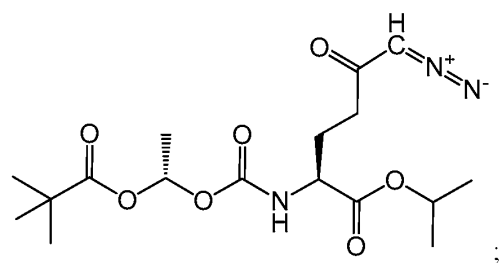
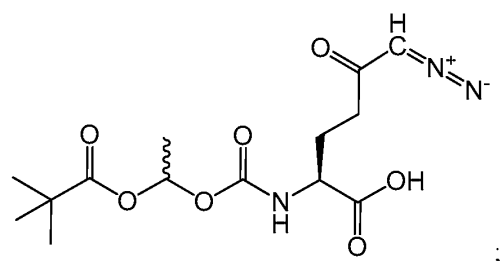
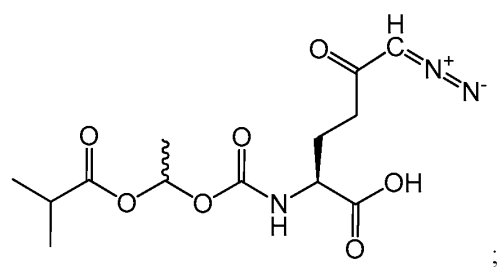
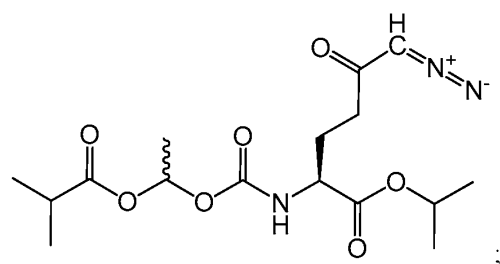
- (i) m is 1;
 R_3 is H; and
 R_4 is methyl, iPr, or aryl;
- (ii) m is 1;
 R_3 and R_4 are each H; or

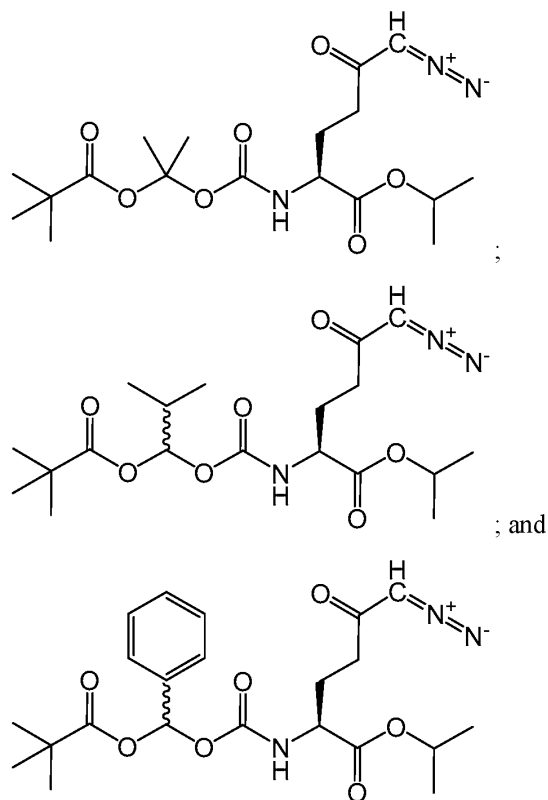
- (iii) m is 1;
R₃ and R₄ are each methyl.

20. The prodrug of claim 19, wherein the compound of formula (I) is selected from the group consisting of:

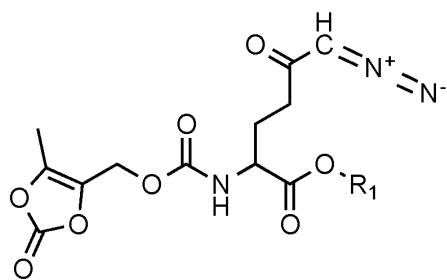






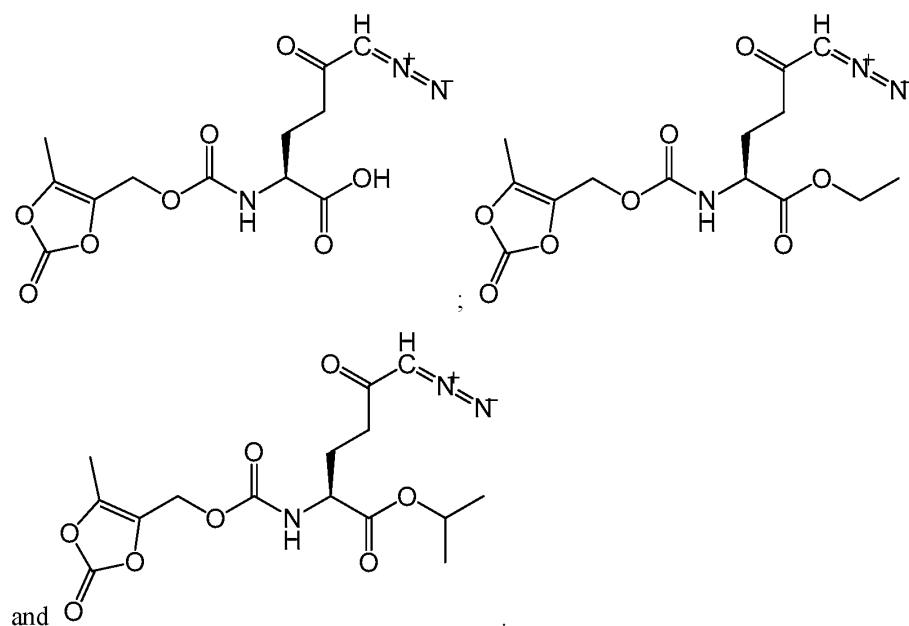


21. The prodrug of claim 11, wherein the compound of formula (I) is of formula:



, wherein R₁ is as defined above in claim 1.

22. The prodrug of claim 21, wherein the compound of formula (I) is selected from the group consisting of:



23. The prodrug of claim 11, wherein R_2 is $-C(=O)-Y-(CR_3R_4)_m-NR_5R_6$;
wherein

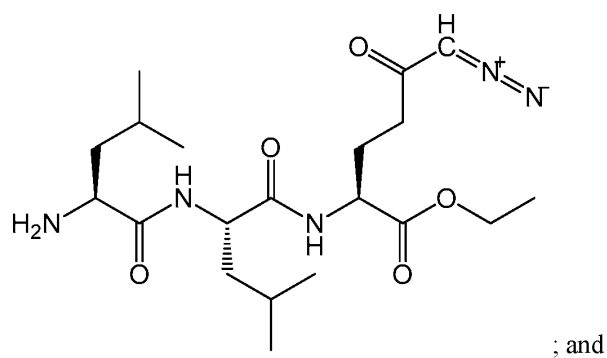
Y is a bond;

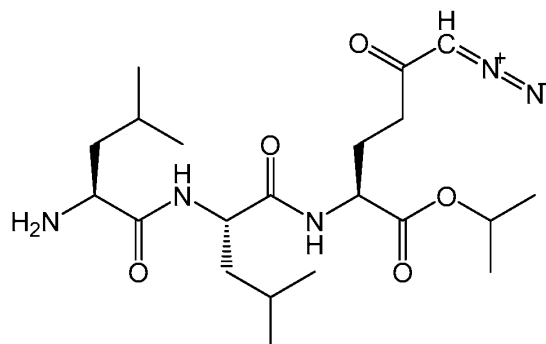
each m is 1;

each R_5 is H;

each R_6 is independently H or $-C(=O)-(CR_3R_4)_m-NR_5R_6$.

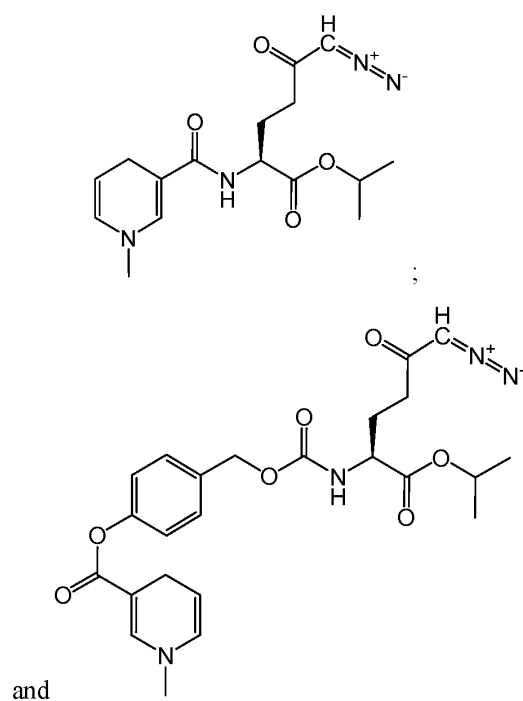
24. The prodrug of claim 23, wherein the compound of formula (I) is
selected from the group consisting of:





25. The prodrug of claim 11, wherein R_2 is $-C(=O)-Ar$,
or $-C(=O)-Y-(CR_3R_4)_m-Ar-O-C(=O)-Ar$.

26. The prodrug of claim 25, wherein the compound of formula (I) is
selected from the group consisting of:



27. The prodrug of claim 11, wherein R_2 is $-C(=O)-Y-(CR_3R_4)_m-Ar-$
 NR_5R_6 ; wherein:

Y is 0;

each m is independently 0, 1, or 3;

each R₃ is independently H, C₁-C₆ alkyl, or -(CR₃R₄)_m-NR₅R₆;

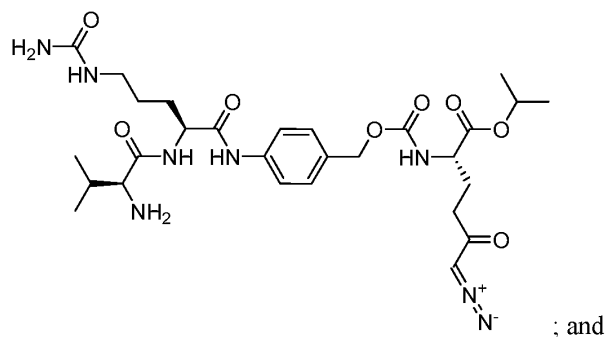
each R_4 is H;

each R₅ is independently H, -C(=O)-(CR₃R₄)_m, -C(=O)-NR₅R₆, or -C(=O)-(CR₃R₄)_m-NR₅R₆;

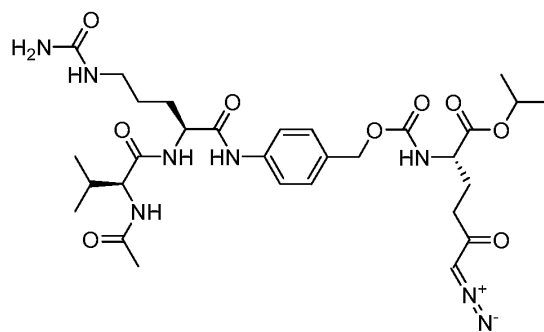
each R_6 is H.

each R_6 is H.

28. The prodrug of claim 27, wherein the compound of formula (I) is selected from the group consisting of:



; and



29. The prodrug of claim 11, wherein R₂ is -C(=O)-Y-(CR₃R₄)_m-NR₅R₆,
wherein:

Y is a bond;

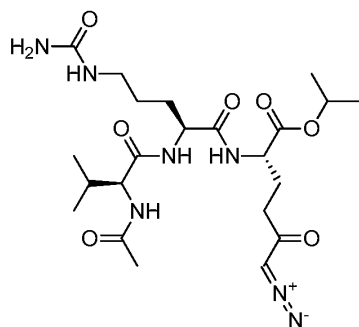
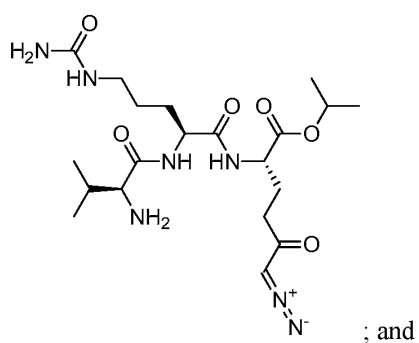
each m is independently 1, 2 or 3;

each R₃ is independently H, C₁-C₆ alkyl, or -(CR₃R₄)_m-NR₅R₆;

each R_4 is H;

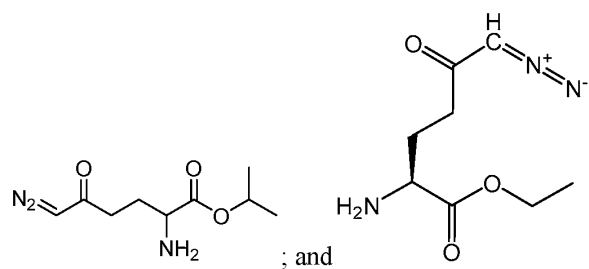
each R_5 is independently H, $-C(=O)-(CR_3R_4)_m$, $-C(=O)-(NR_5R_6)$, or $-C(=O)-(CR_3R_4)_m-NR_5R_6$;
 each R_6 is H.

30. The prodrug of claim 29, wherein the compound of formula (I) is selected from the group consisting of:



31. The prodrug of claim 11, wherein R_2 is H and R_1 is selected from the group consisting of alkyl and substituted alkyl.

32. The prodrug of claim 31, wherein the compound of formula (I) is selected from the group consisting of:



33. A pharmaceutical composition comprising a compound of formula (I),
and a pharmaceutically acceptable carrier, diluent, or excipient.