

SELF-HYDROLYZING MALEIMIDES FOR BIOCONJUGATION

FIELD

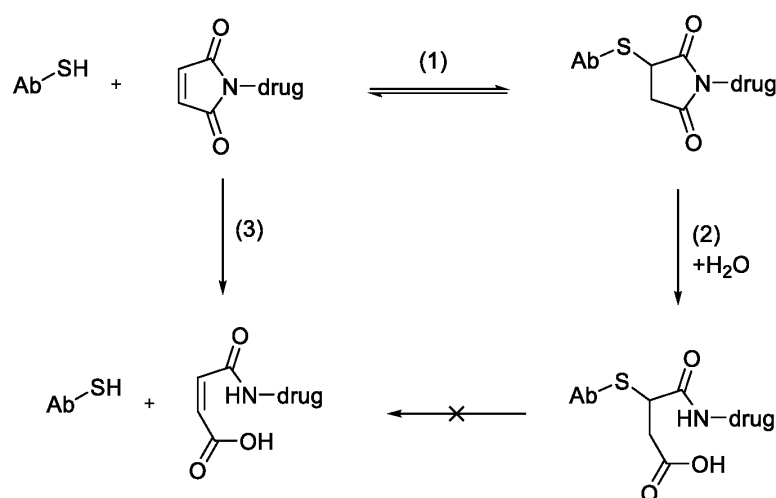
[0001] The present disclosure relates to novel compounds comprising self-hydrolyzing maleimides for the reaction with molecules including a thiol functional group in a thiol conjugation reaction. The present disclosure also relates to novel compounds comprising self-hydrolyzing maleimides for bioconjugation to antibodies or portions of antibodies.

BACKGROUND

[0002] Biological molecules, such as antibodies, frequently include one or more thiol functional groups in cysteine amino acids. Thiol functional groups from cysteine amino acids are frequently used to attach drugs to antibodies, thereby forming an antibody-drug conjugates (ADCs). The drug portion of the ADC may include a maleimide functional group, which reacts in a bioconjugation reaction with the thiol functional group from the biological molecule to form a thiosuccinimide link between the antibody and the drug portion. This thiosuccinimide bioconjugation reaction occurs rapidly under physiological conditions, attains nearly quantitative conjugation without a large excess of either species, and can be applied to a vast array of molecules of biological interest. See Nature Biotechnology, 2014, 32, 1059-1062.

[0003] However, the formation of thiosuccinimide is reversible, with maleimide elimination occurring slowly under biologically relevant conditions. See Nature Biotechnology, 2014, 32, 1059-1062. Thus, many ADCs may experience measurable drug loss during prolonged circulation in the body, leading to diminished activity. See Nature Biotechnology, 2014, 32, 1059-1062. However, the elimination of the maleimide functional group can be mitigated if the thiosuccinimide instead undergoes a ring-opening hydrolysis reaction that results in a conjugation link that is no longer subject to maleimide elimination. . See Nature Biotechnology, 2014, 32, 1059-1062. See Also Formula A.

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[0004]

[0005] Formula A. Self-Hydrolyzing Maleimide Functional Groups

[0006] An amine adjacent to the maleimide group may induce hydrolysis of the thiosuccinimide link after the antibody-maleimide conjugate forms. This maleimide group induced hydrolysis is an example of self-hydrolysis. The hydrolysis kinetics generally correlate with the distance between the amine and the maleimide, where the closer the amine is to the maleimide, the faster the rate of self-hydrolysis. See Nature Biotechnology, 2014, 32, 1059-1062.

[0007] Thus, there is a need for novel compounds comprising a maleimide functional group that (1) undergoes a bioconjugation reaction with thiol functional groups on biological molecules, and (2) undergoes a rapid self-hydrolysis of the formed thiosuccinimide to (3) prevent any loss of maleimide functional groups under desirable physiological conditions. See Formula A.

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SUMMARY

[0008] Disclosed herein are novel compounds comprising a maleimide functional group that (1) undergoes a bioconjugation reaction with thiol functional groups, such as those found on biological molecules, and (2) undergoes a rapid self-hydrolysis of the formed thiosuccinimide

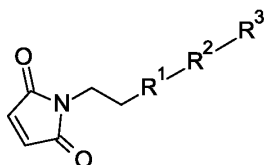
[0009] Also disclosed herein are self-hydrolyzing maleimide-containing compounds which exhibit nearly quantitative rates of conjugation and self-hydrolysis under desirable physiological conditions. Also disclosed herein are salts and/or pharmaceutically

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acceptable salts of these novel compounds. Also disclosed herein are methods of preparing an antibody-maleimide conjugate, and methods of making self-hydrolyzing maleimide-containing compounds.

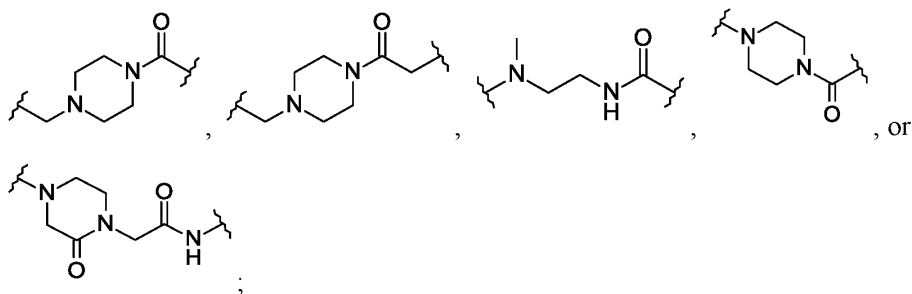
[0010] Disclosed herein is a compound of formula:



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or a salt thereof,

wherein R¹ is

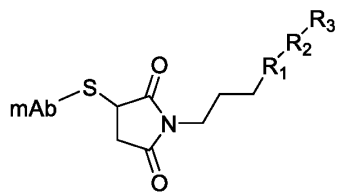


10 R² is selected from the group consisting of PEG_n, a bond, and a peptide, or a combination thereof;

n is 1 to 50; and

R³ is a -phenyl-tetrazine group, or a ligation group, wherein the tetrazine is optionally substituted with methyl.

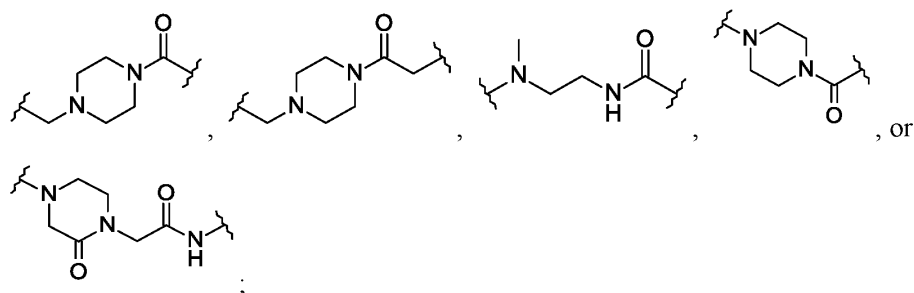
15 [0011] Also disclosed herein, is a conjugate of the formula:



or a salt thereof,

wherein R¹ is

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R^2 is selected from the group consisting of PEG_n , a bond, and a peptide, or a combination thereof;

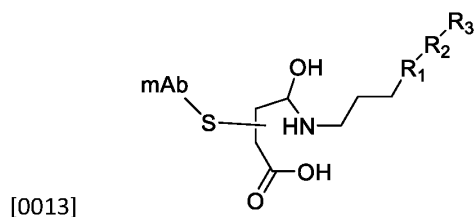
5 n is 1 to 50;

R^3 is a -phenyl-tetrazine group, or a ligation group, wherein the tetrazine is optionally substituted with methyl;

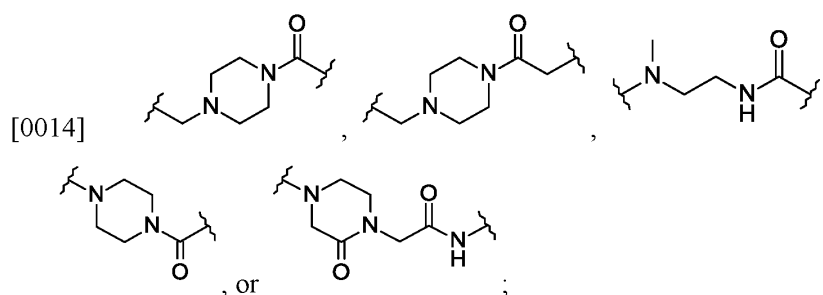
mAb is a monoclonal antibody; and

S is a sulfur atom from a cysteine residue on the monoclonal antibody.

10 [0012] Also disclosed herein is conjugate of the formula:



or a salt thereof, wherein R^1 is



15 R^2 is selected from the group consisting of PEG_n , a bond, and a peptide, or a combination thereof;

n is 1 to 50;

R^3 is a -phenyl-tetrazine group, or a ligation group, wherein the tetrazine is optionally substituted with methyl;

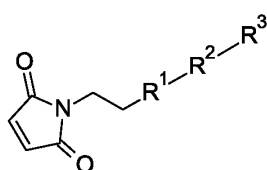
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mAb is a monoclonal antibody; and

S is a sulfur atom from a cysteine residue on the monoclonal antibody.

[0015] In an aspect, disclosed herein are compounds of Formula I, or salts thereof,
 5 methods of making compounds of Formula I, or salts thereof, and methods of using
 compounds of Formula I or salts thereof, are disclosed.

In an aspect, disclosed herein is a compound of Formula I:

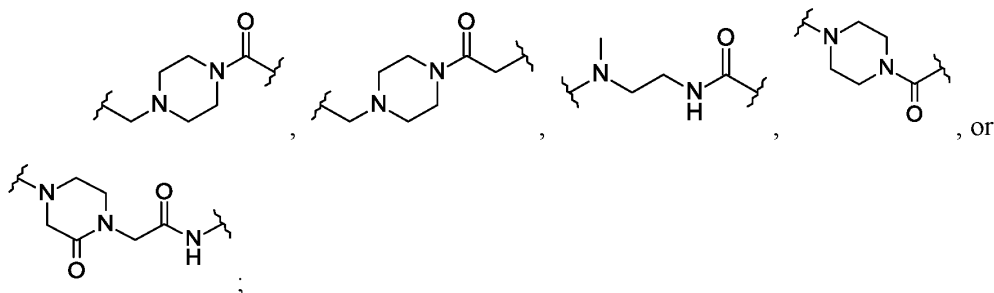


Formula I,

10

or a salt thereof,

wherein R¹ is



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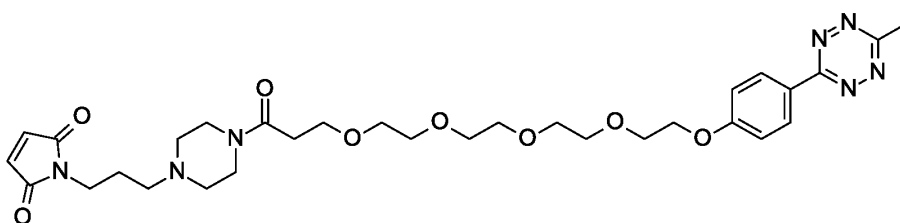
R² is selected from the group consisting of PEG_n, a bond, and a peptide, or a
 combination thereof;

n is 1 to 50;

R³ is a -phenyl-tetrazine group, or a ligation group, wherein the tetrazine is
 optionally substituted with methyl.

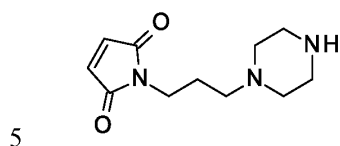
In an aspect, disclosed herein is a compound,

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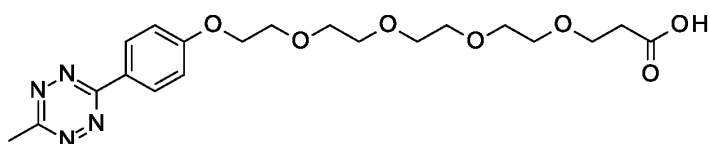


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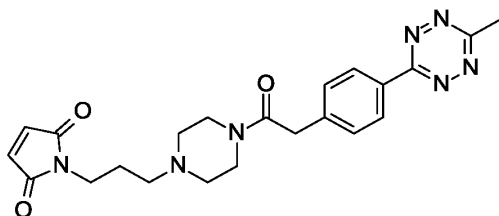
1-[3-[4-[3-[2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanoyl]piperazin-1-yl]propyl]pyrrole-2,5-dione ("Compound A") or a salt thereof, obtained by combining 1-(3-piperazin-1-ylpropyl)pyrrole-2,5-dione,



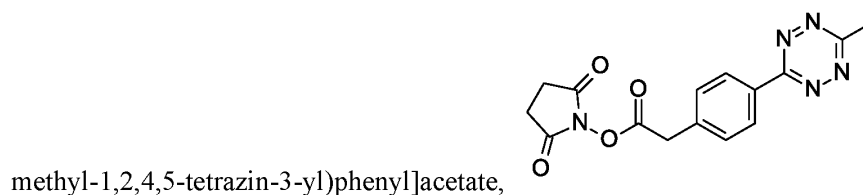
with 3-[2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanoic acid ("methyltetrazine-PEG4-acid"),



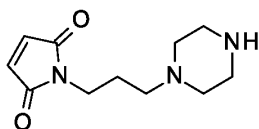
In an aspect, disclosed herein is 1-[3-[4-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]acetyl]piperazin-1-yl]propyl]pyrrole-2,5-dione ("Compound B"), which has the formula:



or a salt thereof, obtained by combining (2,5-dioxopyrrolidin-1-yl) 2-[4-(6-



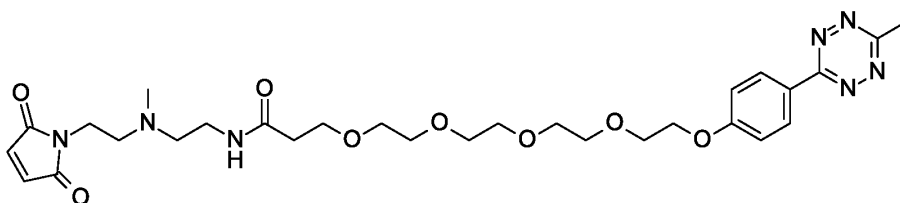
15 1-(3-piperazin-1-ylpropyl)pyrrole-2,5-dione,



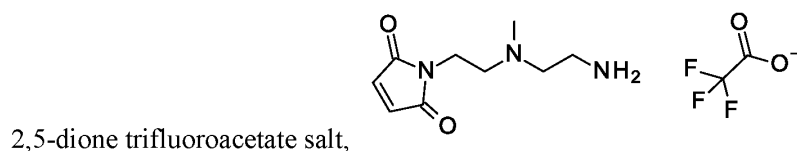
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and a base, in the presence of a solvent and to give Compound B.

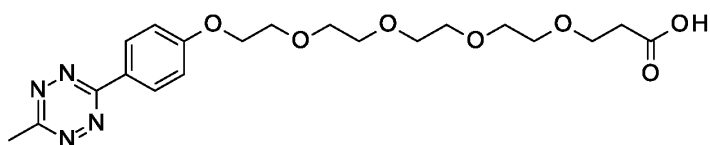
In an aspect, disclosed herein is N-[2-[2-(2,5-dioxopyrrol-1-yl)ethyl-methyl-amino]ethyl]-3-[2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanamide ("Compound C"), which has the following formula:



or a salt thereof, obtained by combining 1-[2-[2-aminoethyl(methyl)amino]ethyl]pyrrole-

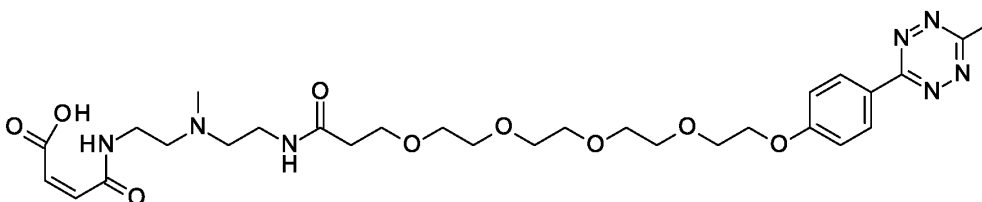


10 methyltetrazine-PEG4-acid,



and an amide coupling reagent, and a base in a solvent.

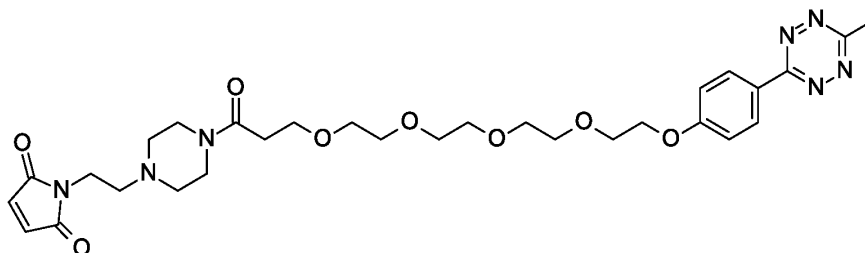
In an alternate aspect, Compound C or a salt thereof, is obtained by combining (Z)-4-[2-[methyl-[2-[3-[2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanoylamino]ethyl]amino]ethylamino]-4-oxo-but-2-enoic acid,



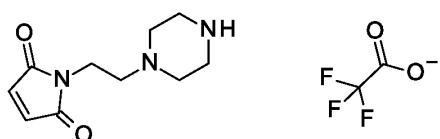
, acetate salt, and acetic anhydride.

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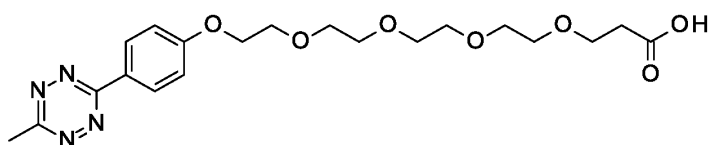
In an aspect, disclosed herein is -[3-[2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanoyl]piperazin-1-yl]ethyl]pyrrole-2,5-dione ("Compound D"):



- 5 1-[2-[4 or a salt thereof, obtained by combining 1-(2-piperazin-1-ylethyl)pyrrole-2,5-dione trifluoroacetate,

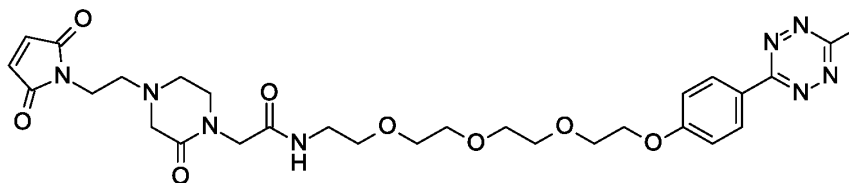


methyltetrazine-PEG4-acid,



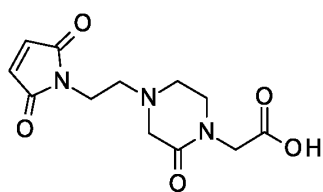
- 10 an amide coupling reagent, and a base, to a solvent.

In an aspect, disclosed herein is 2-[4-[2-(2,5-dioxopyrrol-1-yl)ethyl]-2-oxopiperazin-1-yl]-N-[2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy]ethoxy]ethoxy]ethoxy]ethyl]acetamide ("Compound E") which has the following formula:

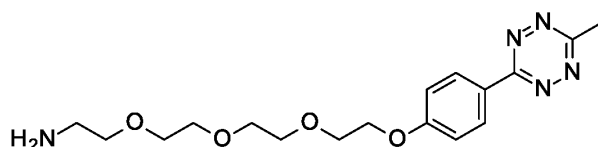


- 15 or a salt thereof, obtained by combining 2-[4-[2-(2,5-dioxopyrrol-1-yl)ethyl]-2-oxopiperazin-1-yl]acetic acid,

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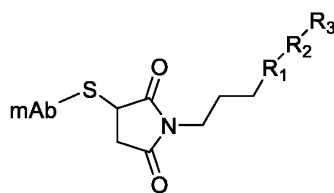


and 2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy]ethoxy]ethoxy]ethoxy]ethanamine (“methyltetrazine-PEG4-amine”),



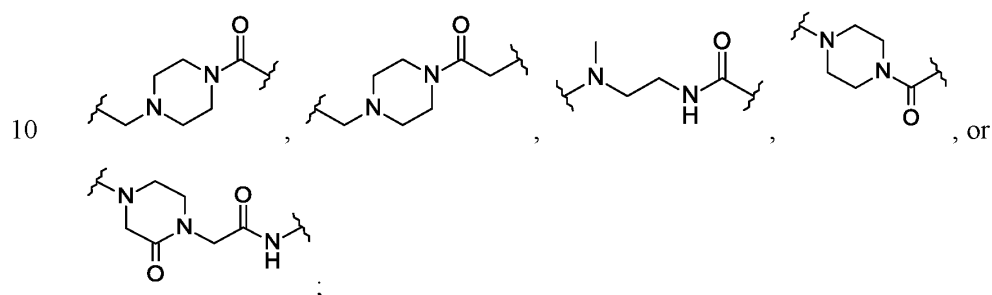
5 an amide coupling reagent, an amide coupling solvent, and a base.

In an aspect, disclosed herein is a conjugate of the formula:



or a salt thereof,

wherein R¹ is



R² is selected from the group consisting of PEG_n, a bond, and a peptide, or a combination thereof;

n is 1 to 50;

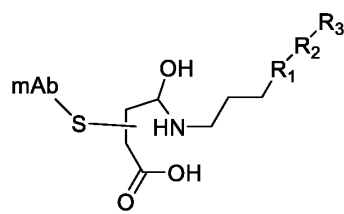
15 R³ is a -phenyl-tetrazine group, or a ligation group, wherein the tetrazine is optionally substituted with methyl;

mAb is a monoclonal antibody; and

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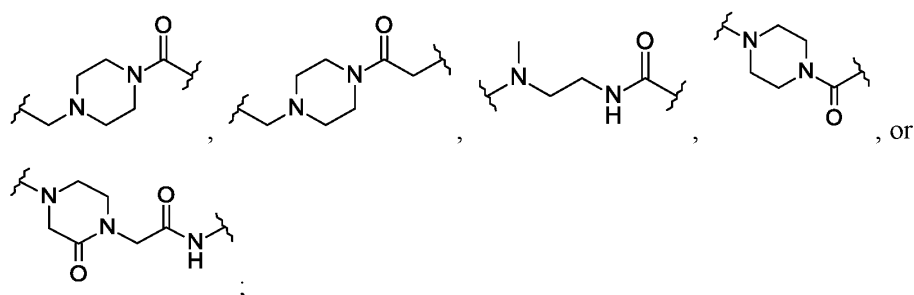
S is a sulfur atom from a cysteine residue on the monoclonal antibody.

In an aspect, disclosed herein is a conjugate of the formula:



or a salt thereof,

5 wherein R¹ is



R² is selected from the group consisting of PEG_n, a bond, and a peptide, or a combination thereof;

10 n is 1 to 50;

R³ is a -phenyl-tetrazine group, or a ligation group, wherein the tetrazine is optionally substituted with methyl;

mAb is a monoclonal antibody; and

S is a sulfur atom from a cysteine residue on the monoclonal antibody.

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DETAILED DESCRIPTION

Definitions

The term “salt” as used herein refers to a salt of a compound. The term “pharmaceutically acceptable salt” as used herein refers to a salt of a compound considered to be acceptable for clinical and/or veterinary use. Examples of pharmaceutically acceptable salts and common methodology for preparing them can be found in “Handbook of Pharmaceutical Salts: Properties, Selection and Use” P. Stahl, *et*

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al., 2nd Revised Edition, Wiley-VCH, 2011 and S.M. Berge, *et al.*, "Pharmaceutical Salts", *Journal of Pharmaceutical Sciences*, 1977, 66(1), 1-19.

A compound of Formula I may be readily converted to and may be isolated as a salt, including a pharmaceutically acceptable salt. Salt formation can occur upon the addition of a pharmaceutically acceptable acid to form the acid addition salt. Salts can also form simultaneously upon deprotection of a nitrogen or oxygen, *i.e.*, removing the protecting group. Examples, reactions and conditions for salt formation can be found in Gould, P.L., "Salt selection for basic drugs," *International Journal of Pharmaceutics*, 33: 201-217 (1986); Bastin, R.J., *et al.* "Salt Selection and Optimization Procedures for Pharmaceutical New Chemical Entities," *Organic Process Research and Development*, 4: 427-435 (2000); and Berge, S.M., *et al.*, "Pharmaceutical Salts," *Journal of Pharmaceutical Sciences*, 66: 1-19, (1977). In an embodiment, acceptable counterions could include trifluoroacetate, or chloride.

Table 1: Abbreviations and definitions

Term	Definition
ACN	acetonitrile
AMPSO	N-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid
aq	aqueous
base	substance that reacts with an acid
BOC / Boc	butyloxy carbonyl
C18	octadecylsilane
DCM	dichloromethane
deBoc reagent	N-Boc deprotection reagent, typically any strong acids, such as TFA or HCl/dioxane
DMF	N,N-dimethylformamide
DMSO	dimethylsulfoxide
drying agent	a substance that promotes drying
EtOAc	ethyl acetate
HPLC	high performance liquid chromatography
Hunig's base	N,N-diisopropylethylamine
IPA	isopropanol

Term	Definition
LCMS	liquid chromatography mass spectrometry
MeOH	methanol
min	minute(s)
MS	mass spectrometry
MTBE	methyl tert-butyl ether
<i>m/z</i>	mass-to-charge ratio
Na ₄ P ₂ O ₇	Sodium pyrophosphate
PEG	polyethylene glycol
PEG#	a single polyethylene glycol or a chain of polyethylene glycols, wherein # is the number of PEG monomers. For example, PEG4 is a chain of 4 PEG monomers.
peptide	a moiety including two or more amino acids, or derivatives thereof, linked in a chain, the carboxyl group of at least one amino acid being joined to the amino group of at least another amino acid
RP-HPLC	reverse-phase high performance liquid chromatography
RP-LCMS	reverse-phase liquid chromatography mass spectrometry
rt	room temperature
satd	saturated
solvent	substance that dissolves a solute
SCX	strong cation exchange
TCEP	tris(2-carboxyethyl)phosphine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
Triple phosphate buffer	combinations of PO ₄ , HPO ₄ , and H ₂ PO ₄ anions, typically using sodium or potassium cations
Tris	tris(hydroxymethyl)aminomethane

Table 2: Amide coupling reagent Abbreviations and definitions

Term	Definition
ACTU	(2-(6-chloro-1-H-benzotriazol-1-yl)-1,1,3,3-tetramethylaminium) hexachloroantimonate
AOMP	5-(7-azabenzotriazol-1-yloxy)-3,4-dihydro-1-methyl-2H-pyrrolidium hexachloroantimonate
AOP	(7-azabenzotriazol-1-yl)oxytris(dimethylamino)phosphonium hexafluorophosphate

BBC	benzotriazoloxo-bis(pyrrolidino)carbonium hexafluorophosphate
BDDC	bis[[4-(2,2-dimethyl-1,3-dioxolyl)]methyl]carbodiimide
BDMP	5-(1H-benzotriazol-1-yloxy)-3,4-dihydro-1-methyl-2H-pyrrolidium hexachloroantimonate
BDP	benzotriazol-1-yl diethylphosphate
BEC	N-tert-butyl-N'-ethylcarbodiimide
BEMT	2-bromo-3-ethyl-4-methylthiazolium tetrafluoroborate
BEP	2-bromo-1-ethylpyridinium tetrafluoroborate
BEPH	2-bromo-1-ethylpyridinium hexachloroantimonate
4,5-B(HATU)	N-[(dimethylamino)(3H-1,2,3-triazolo[4,5-c]isoquinolin-3-yloxy)-N-methylmethanaminium hexafluorophosphate
5,6-B(HATU)	1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]quinolinium hexafluorophosphate-3-oxide
BIODPP	diphenyl benzo[d]isoxazol-3-ylphosphonate
BMC	N-tert-butyl-N'-methylcarbodiimide
BMMP	1-(1-(1H-benzo[d][1,2,3]triazol-1-yloxy)ethylidene)pyrrolidinium hexachloroantimonate
BMP-Cl	N,N'-bismorpholinophosphonic chloride
BMTB	2-bromo-3-methyl-4-methylthiazolium bromide
BOI	2-(benzotriazol-1-yl)oxy-1,3-dimethylimidazolidinium hexafluorophosphate
BOMI	benzotriazol-1-yloxy-N,N-dimethylmethaniminium hexachloroantimonate
BOP	benzotriazolyl-N-oxytrisdimethylaminophosphonium hexafluorophosphate
BOP-Cl	N,N'-bis(2-oxo-3-oxazolidinyl)phosphinic chloride
BPMP	1-(1H-benzotriazol-1-yloxy)phenylmethylene pyrrolidinium hexachloroantimonate
BroP	bromotris(dimethylamino)phosphonium hexafluorophosphate
BTC	(bis(trichloromethyl) carbonate, "triphosgene")
BTFFH	bis(tetramethylene)fluoroformamidinium hexafluorophosphate
CBDO	2-chlorobenzo[d][1,3]dioxol-1-ium hexachloroantimonate
CBMIT	1,10-carbonylbis(3-methylimidazolium) triflate
CDI	carbonyldiimidazole
CDMS	chlorodimethylsulfonium hexachloroantimonate

CDMT	2-chloro-4,6-dimethoxy-1,3,5-triazine
CDTP	2-chloro-1,3-dimethyl-3,4,5,6-tetrahydropyrimidin-1-ium perchlorate
CIP	2-chloro-1,3-dimethylimidazolidinium hexafluorophosphate
CloP	chlorotris(dimethylamino)phosphonium hexafluorophosphate
CMMM	chloro(4-morpholino)methylene morpholinium hexafluorophosphate
COMU	ethyl 2-cyano-2-(hydroxyimino)acetate
CPMA	(chlorophenylthiomethylene)dimethylammonium chloride
CPDT	2-chloro-5-phenyl-1,3-dithiol-1-ium hexachloroantimonate
Cpt-Cl	1-oxo-chlorophospholane
DAST	diethylaminosulfur trifluoride
DCC	dicyclohexylcarbodiimide
DCIH	1,3-dimethyl-2-chloro-4,5-dihydro-1H-imidazolium hexafluorophosphate
DCMT	2,4-dichloro-6-methoxy-1,3,5-triazine
DEBP	diethyl-2-(3-oxo-2,3-dihydro-1,2-benzisosulfonazolyl)phosphonate
DECP	diethylcyanophosphonate
DEFFH	1,2-diethyl-3,3-tetramethylenefluoroformamidinium hexafluorophosphate
Deoxo-Fluor	bis(2-methoxyethyl)aminosulfur trifluoride
DEPB	diethyl phosphorobromidate
DEPBO	N-diethoxyphosphorylbenzoxazolone
DEPBT	3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one
DEPC	diethyl phosphorochloridate
DepOAt	3H-[1,2,3]triazolo[4,5-b]pyridin-3-yl diethyl phosphate
DepOBt	diethoxyphosphinyloxybenzotriazole
DepODhbt	diethyl 4-oxobenzo[d][1,2,3]triazin-3(4H)-yl phosphate
DFIH	1,3-dimethyl-2-fluoro-4,5-dihydro-1H-imidazolium hexafluorophosphate
DIC	diisopropylcarbodiimide
DMC-Cl	2-chloro-1,3-dimethylimidazolinium chloride
DMCH	N-(chloro(morpholino)methylene)-N-methylmethanaminium hexafluorophosphate
DMFFH	1,2-dimethyl-3,3-tetramethylenefluoroformamidinium hexafluorophosphate

DMFH	N-(fluoro(morpholino)methylene)-N-methylmethanaminium hexafluorophosphate
DmppOAt	1-(2,8-dimethylphenoxaphosphinyloxy)-7-azabenzotriazole
DMTMM	4-(4,6-dimethoxy[1,3,5]triazin-2-yl)-4-methylmorpholinium chloride
DOMP	5-(3',4'-dihydro-4'-oxo-1',2',3'-benzotriazin-3'-yloxy)-3,4-dihydro-1-methyl 2H-pyrrolidium hexachloroantimonate
DOPBO	N-(2-oxo-1,2,3-dioxaphosphorinanyl)benzoxazolone
DOPBT	3-[O-(2-oxo-1,2,3-dioxaphosphorinanyl)oxy]-1,2,3-benzotriazin-4(3H)-one
DPOOP	diphenyl-2-oxo-3-oxazolinylphosphonate
Dpop-Cl	diphenyl phosphorochloridate
DpopOAt	1-(diphenoxyphosphoryloxy)-7-azabenzotriazole
DpopOBt	1-(diphenoxyphosphoryloxy)benzotriazole
DpopODhbt	3-(diphenoxyphosphinyloxy)-3,4-dihydro-4-oxo-1,2,3-benzotriazine
DPP	diphenylphosphite
DPPA	diphenylphosphoryl azide
Dpp-Cl	diphenylphosphinic chloride
DPTC	O,O'-di(2-pyridyl)thiocarbonate
DPTF	2,2-dichloro-5-(2-phenylethyl)-4-(trimethylsilyl)-3-furanone
DtpOAt	1-[di(O-tolyl)phosphinyloxy]-7-azabenzotriazole
DtpOBt	1-[di(O-tolyl)phosphinyloxy]benzotriazole
DtpODhbt	3-di(O-tolyl)phosphinyloxy]-3,4-dihydro-4-oxo-1,2,3-benzotriazine
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EEDQ	N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline
ENDPP	phosphoric acid 3,5-dioxo-10-oxa-4-azatricyclo[5.2.1.0 ^{2,6}]dec-8-en-4-yl ester diphenyl ester
FDMP	3,5-bis(trifluoromethyl)phenyl diphenylphosphinate
FDPP	pentafluorophenyl diphenyl phosphinate
FEP	2-fluoro-1-ethylpyridinium tetrafluoroborate
FEPH	2-fluoro-1-ethylpyridinium hexachloroantimonate
FOMP	5-(pentafluorophenyl)-3,4-dihydro-1-methyl-2H-pyrrolidium hexachloroantimonate
HAE2PipU	O-(1H-1,2,3-triazolo[4,5-b]pyridin-1-yl)-1,1-diethyl-3,3-pentamethylenuronium hexafluorophosphate

HAE2PyU	O-(1H-1,2,3-triazolo[4,5-b]pyridin-1-yl)-1,1-diethyl-3,3-tetramethylenuronium hexafluorophosphate
HAMDU	O-(7-azabenzotriazol-1-yl)-1,3-dimethyl-1,3-dimethylenuronium hexafluorophosphate
HAM2PipU	O-(1H-1,2,3-triazolo[4,5-b]pyridin-1-yl)-1,1-dimethyl-3,3-pentamethylenuronium hexafluorophosphate
HAM2PyU	O-(1H-1,2,3-triazolo[4,5-b]pyridin-1-yl)-1,1-dimethyl-3,3-tetramethylenuronium hexafluorophosphate
HAMTU	O-(7-azabenzotriazol-1-yl)-1,1,3,3-bis(pentamethylene)uronium hexafluorophosphate
HAPipU	O-(7-azabenzotriazol-1-yl)-1,1,3,3-bis(pentamethylene)uronium hexafluorophosphate
HAPyTU	S-(7-azabenzotriazol-1-yl)-1,1,3,3-bis(tetramethylene)thiuronium hexafluorophosphate
HAPyU	1-(1-pyrrolidinyl-1H-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene)pyrrolidinium hexafluorophosphate N-oxide
HATeU	O-(1H-1,2,3-triazolo[4,5-b]pyridin-1-yl)-1,1,3,3-tetraethyluronium hexafluorophosphate
HATU	O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HBE2PipU	O-(1H-benzotriazol-1-yl)-1,1-diethyl-3,3-pentamethylenuronium hexafluorophosphate
HBE2PyU	O-(1H-benzotriazol-1-yl)-1,1-diethyl-3,3-tetramethylenuronium hexafluorophosphate
HBMDU	O-(benzotriazol-1-yl)-1,3-dimethyl-1,3-dimethylenuronium hexafluorophosphate
HBMP	1H-benzo[d][1,2,3]triazol-1-ylmethanesulfonate
HBM2PipU	O-(1H-benzotriazol-1-yl)-1,1-dimethyl-3,3-pentamethylenuronium hexafluorophosphate
HBM2PyU	O-(1H-benzotriazol-1-yl)-1,1-dimethyl-3,3-tetramethylenuronium hexafluorophosphate
HBPipU	O-(benzotriazol-1-yl)-1,1,3,3-bis(pentamethylene)uronium hexafluorophosphate
HBSP	1H-benzo[d][1,2,3]triazol-1-ylbenzenesulfonate
HBTeU	O-(1H-benzotriazol-1-yl)-1,1,3,3-tetraethyluronium hexafluorophosphate
HBTU	O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HCTU	(2-(6-chloro-1-H-benzotriazol-1-yl)-1,1,3,3-tetramethylaminium) hexafluorophosphate
HCSCP	6-chloro-1H-benzo[d][1,2,3]triazol-1-yl-4-chlorobenzenesulfonate
HCSP	6-chloro-1H-benzo[d][1,2,3]triazol-1-ylbenzenesulfonate
HDATU	(bis(dimethylamino)methyl)(4-oxopyrido[3,2-d][1,2,3]triazin-3(4H)-yl)oxonium hexafluorophosphate

HDADU	(bis(dimethylamino)methyl)(4-oxopyrido[3,2-d]pyrimidin-3(4H)-yl)oxonium hexafluorophosphate
HDAPyU	1-((4-oxopyrido[3,2-d][1,2,3]triazin-3(4H)-yloxy)(pyrrolidin-1-yl)methylene)pyrrolidinium hexafluorophosphate
HDMA	1-((dimethylamino)(morpholino)methylene)-1H-[1,2,3]triazolo[4,5-b]pyridinium hexafluorophosphate 3-oxide
4-HDMA	3-((dimethylamino)(morpholino)methylene)-1H-[1,2,3]triazolo[4,5-b]pyridinium hexafluorophosphate 1-oxide
HDMB	1-((dimethylamino)(morpholino)methylene)-1H-benzotriazolium hexafluorophosphate 3-oxide
HDMCB	6-chloro-1-((dimethylamino)(morpholino)methylene)-1H-benzotriazolium hexafluorophosphate 3-oxide
HDMFB	6-trifluoromethyl-1-((dimethylamino)(morpholino)methylene)-1H-benzotriazolium hexafluorophosphate 3-oxide
HDMPfp	1-((dimethylamino)(morpholino))oxypentafluorophenyl metheniminium hexafluorophosphate
HDMS	1-((dimethylamino)(morpholino))oxypyrrolidine-2,5-dione methanaminium hexafluorophosphate
HDPyU	1-((4-oxobenzo[d][1,2,3]triazin-3(4H)-yloxy)(pyrrolidin-1-yl)methylene)pyrrolidinium hexafluorophosphate
HDTMA	1-((dimethylamino)(thiomorpholino)methylene)-1H-[1,2,3]triazolo[4,5-b]pyridinium hexafluorophosphate 3-oxide
HDTMB	1-((dimethylamino)(thiomorpholino)methylene)-1H-benzotriazolium hexafluorophosphate 3-oxide
HDTU	O-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	1-hydroxy-1H-benzotriazole
HODhat	3-hydroxy-4-oxo-3,4-dihydro-5-azabenzotriazine
HODhbt	3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine
HODT	S-(1-oxido-2-pyridinyl)-1,3-dimethyl-1,3-trimethylenethiouronium
HONB	2-(5-norbornene-2,3-dicarboximide)
HOPfp	pentafluorophenol
HPyOPfp	N,N,N',N'-bis(tetramethylene)-O-pentafluorophenyluronium hexafluorophosphate
HPySPfp	1-((perfluorophenylthio)(pyrrolidin-1-yl)methylene)pyrrolidinium hexafluorophosphate
HOSu	N-hydroxysuccinimide
HOTT	S-(1-oxido-2-pyridinyl)-1,1,3,3-tetramethylthiouronium hexafluorophosphate
HSTU	O-(N-succimidyl)-N,N,N',N'-bis(tetramethylene)uronium hexafluorophosphate

IIDQ	N-isobutoxycarbonyl-2-isobutoxy-1,2-dihydroquinoline
maleic agent	compound that comprises a dicarboxylic acid or an imide
MPTA	dimethylphosphinothioyl azide
MPT-Cl	dimethylphosphinothioyl chloride
MPTO	3-dimethylphosphinothioyl-2(3H)-oxazolone
NDPP	norborn-5-ene-2,3-dicarboximidodiphenylphosphate
NOP	[(6-nitrobenzotriazol-1-yl)oxy]tris(dimethylaminop)phosphonium hexafluorophosphate
PEC	phenylethylcarbodiimide
PFNB	perfluorophenyl 4-nitrobenzenesulfonate
PIC	phenylisopropylcarbodiimide
PTOC	pyridine-2-thione-N-oxycarbonyl
PyAOP	[(7-azabenzotriazol-1-yl)oxy]tris(pyrrolidino)phosphonium hexafluorophosphate
PyBOP	benzotriazol-1-yloxytri(pyrrolidino)phosphonium hexafluorophosphate
PyBroP	bromotri(pyrrolidino)phosphonium hexafluorophosphate
PyClock	6-chloro-1-hydroxybenzotriazol-1-yl-N-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate
PyCloP	chlorotri(pyrrolidino)phosphonium hexafluorophosphate
PyClopP	chlorobispyrrolidinophenylphosphonium hexachloroantimonate
PyFloP	fluorotri(pyrrolidino)phosphonium hexafluorophosphate
PyCIU	chlorodipyrrolidinocarbenium hexafluorophosphate
PyDAOP	(4-oxopyrido[3,2-d][1,2,3]triazin-3(4H)-yloxy)tripyrrolidin-1-ylphosphonium hexafluorophosphate
PyDOP	[(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)oxy]tris(pyrrolidino)phosphonium hexafluorophosphate
PyDPP	diphenyl 2-oxopyridin-1(2H)-ylphosphonate
PyFOP	[[6-(trifluoromethyl)benzotriazol-1-yl]oxy]tris(pyrrolidino)phosphonium hexafluorophosphate
PyNOP	[(6-nitrobenzotriazol-1-yl)oxy]tris(pyrrolidino)phosphonium hexafluorophosphate
PyPOP	(perfluorophenoxy)tripyrrolidin-1-ylphosphonium
PyTOP	(pyridyl-2-thio)tris(pyrrolidino)-phosphonium hexafluorophosphate
SDPP	2,5-dioxopyrrolidin-1-yl diphenyl phosphate
SMDOP	4-oxobenzo[d][1,2,3]triazin-3(4H)-yl methanesulfonate

SPDOP	4-oxobenzo[d][1,2,3]triazin-3(4H)-yl benzenesulfonate
SOMI	5-(succinimidylloxy)-N,N-dimethylmethaniminium hexachloroantimonate
SOMP	5-(succinimidylloxy)-3,4-dihydro-1-methyl-2H-pyrrolium hexachloroantimonate
T3P	2-propanephosphonic acid anhydride
TATU	O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TAPipU	1-(1-pyrrolidinyl-1H-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene)pyrrolidinium tetrafluoroborate N-oxide
TBFH	N,N,N',N'-tetramethylbromoforamidinium hexafluorophosphate
TBTU	O-benzotriazol-1-yl-1,1,3,3-tetramethyluronium tetrafluoroborate
TCFH	N,N,N',N'-tetramethylchloroforamidinium hexafluorophosphate
TCTU	(2-(6-chloro-1-H-benzotriazol-1-yl)-1,1,3,3-tetramethylaminium) tetrafluoroborate
TDBTU	2-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TEFFH	tetraethylfluoroforamidinium hexafluorophosphate
TFMS-DEP	diethylphenyl(trifluoromethylsulfonyl)phosphoramidate
TFFH	tetramethylfluoroforamidinium hexafluorophosphate
TNTU	2-(5-norbornene-2,3-dicarboximido)-1,1,3,3-tetramethyluronium tetrafluoroborate
TODT	S-(1-oxido-2-pyridinyl)-1,3-dimethyl-1,3-trimethylenethiouronium tetrafluoroborate
TOTT	S-(1-oxido-2-pyridinyl)-1,1,3,3-tetramethylthiouronium tetrafluoroborate
TOTU	O-(cyano(ethoxycarbonyl)methylenamino)-1,1,3,3-tetramethyluronium tetrafluoroborate
TPTU	1-((dimethylamino)(dimethyliminio)methoxy)-2-hydroxypyridinium tetrafluoroborate
TSTU	2-succinimido-1,1,3,3-tetramethyluronium tetrafluoroborate

Table 3: Amide coupling solvent abbreviations and definitions

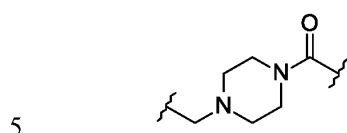
Term	Definition
DCM	dichloromethane
DMF	N,N-dimethylformamide
DMA	dimethylacetamide
NMP	N-methylpyrrolidone
THF	tetrahydrofuran

TBME	tert-butyl methyl ether
CPME	cyclopentylmethyl ether
DMC	dimethylcarbonate
EtOAc	ethyl acetate
IPA	isopropanol
2-MeTHF	2-methyltetrahydrofuran
GVL	γ -valerolactone
Cyrene™	dihydrolevoglucosenone

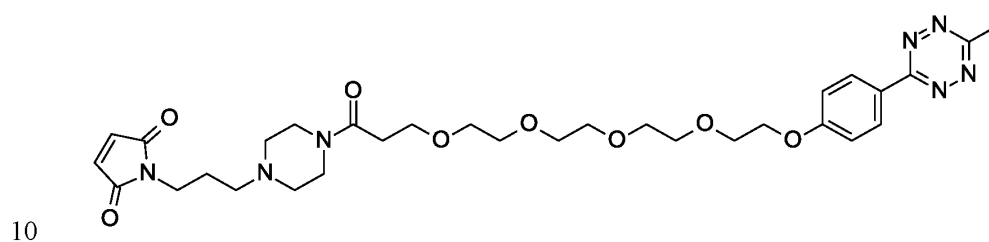
In some embodiments, combinations of amide coupling reagents are used. In some embodiments, combinations of amide coupling solvents are used.

Embodiments of the compounds, and salts, of Formula I

In an embodiment, R^1 is



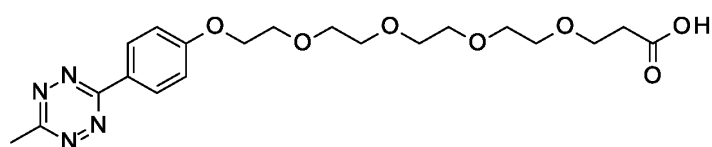
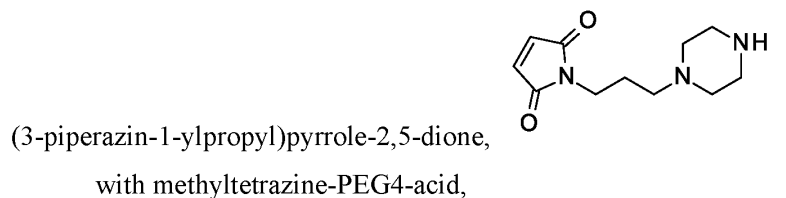
In another embodiment, R^2 is PEG_n , wherein n is 1 to 10. In another embodiment, R^2 is PEG_n , wherein n is 2 to 6. In another embodiment, R^3 is a -phenyl-tetrazine group, wherein the tetrazine is optionally substituted with methyl. In another embodiment, the compound is



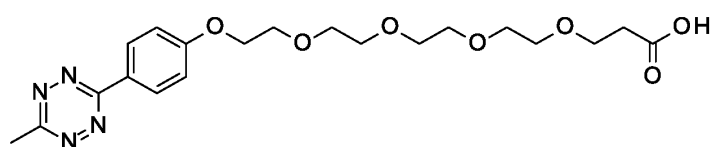
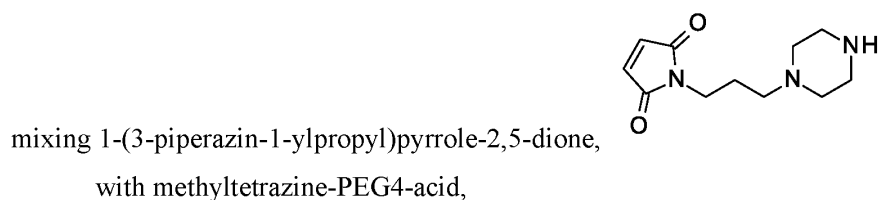
1-[3-[4-[3-[2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanoyl]piperazin-1-yl]propyl]pyrrole-2,5-dione ("Compound A") or a salt thereof. In another embodiment, the compound is Compound A.

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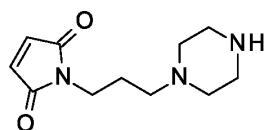
In another embodiment, Compound A, or a salt thereof, is obtained by mixing 1-



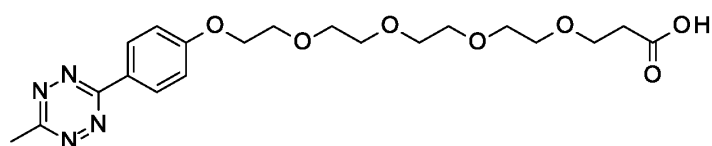
5 in the presence of an amide coupling reagent, an amide coupling solvent, and a base. In another embodiment, Compound A, or a salt thereof, is obtained by the step of



10 in the presence of an amide coupling reagent and a solvent. In some embodiments, Compound A, or a salt thereof, is obtained by the step of mixing 1-(3-piperazin-1-ylpropyl)pyrrole-2,5-dione,



with methyltetrazine-PEG4-acid,

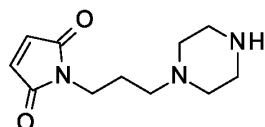


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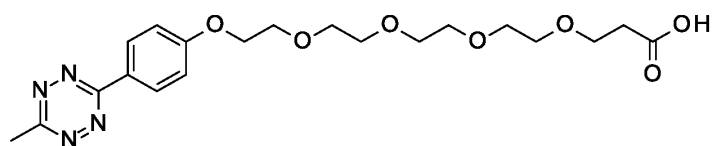
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in the presence of an amide coupling reagent, and a base, in a solvent. In one embodiment, Compound A is obtainable as a free base.

In another embodiment, methods of making Compound A, or salts thereof are disclosed. The methods of making Compound A comprise mixing 1-(3-piperazin-1-ylpropyl)pyrrole-2,5-dione,
 5 ylpropyl)pyrrole-2,5-dione,

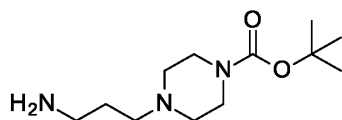


with methyltetrazine-PEG4-acid,

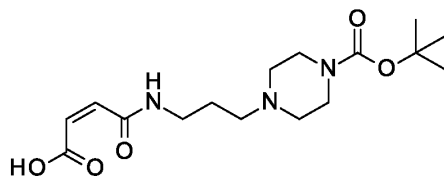


in the presence of an amide coupling reagent and a solvent.

10 In another embodiment, methods of making Compound A are disclosed comprising the step of combining a maleic agent and tert-butyl 4-(3-aminopropyl)piperazine-1-carboxylate,



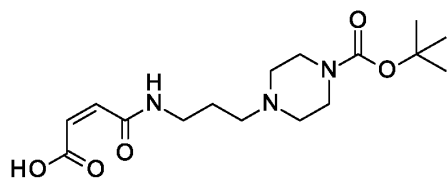
in acetic acid to give (Z)-4-[3-(4-tert-butoxycarbonylpiperazin-1-yl)propylamino]-
 15 4-oxo-but-2-enoic acid,



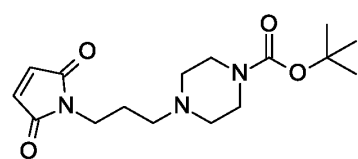
(“Preparation 1 Intermediate 1”). In another embodiment, methods of making Compound A also comprise the step of combining (Z)-4-[3-(4-tert-

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butoxycarbonylpiperazin-1-yl)propylamino]-4-oxo-but-2-enoic acid,

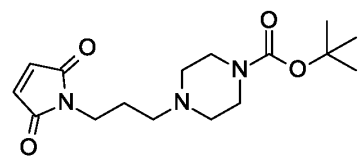


(“Preparation 1 Intermediate 1”), triethylamine, and a drying agent to give tert-butyl 4-[3-(2,5-dioxopyrrol-1-yl)propyl]piperazine-1-carboxylate,



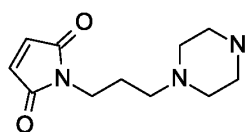
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(“Preparation 1”). In another embodiment, methods of making Compound A also disclose wherein the drying agent comprises 4Å molecular sieves. In another embodiment, methods of making Compound A also comprise the step of combining tert-butyl 4-[3-(2,5-dioxopyrrol-1-yl)propyl]piperazine-1-carboxylate,



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(“Preparation 1”) and a deBoc reagent to give 1-(3-piperazin-1-ylpropyl)pyrrole-2,5-dione,

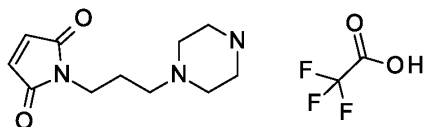


In another embodiment, methods of making Compound A also disclose wherein the deBoc reagent comprises trifluoroacetic acid in DCM, HCl in dioxane, HCl in methanol, or HCl in diethyl ether. In another embodiment, methods of making Compound A also disclose wherein the deBoc reagent comprises trifluoroacetic acid in DCM to give

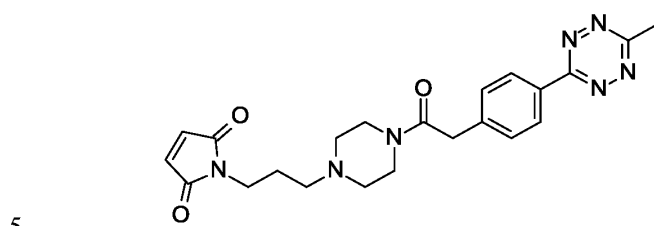
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1-(3-piperazin-1-ylpropyl)pyrrole-2,5-dione trifluoroacetate salt,

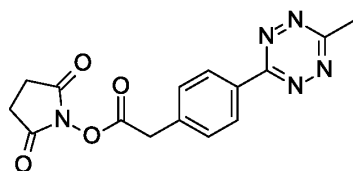


In an embodiment, R^2 is a bond and R^3 is a -phenyl-tetrazine group, wherein the tetrazine is optionally substituted with methyl. In another embodiment, the compound is

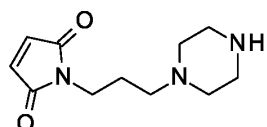


1-[3-[4-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]acetyl]piperazin-1-yl]propyl]pyrrole-2,5-dione ("Compound B") or a salt thereof. In another embodiment, the compound is Compound B.

In another embodiment, Compound B, or a salt thereof, is obtained by combining
10 (2,5-dioxypyrrolidin-1-yl) 2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]acetate,

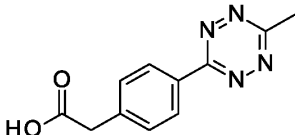


and 1-(3-piperazin-1-ylpropyl)pyrrole-2,5-dione,



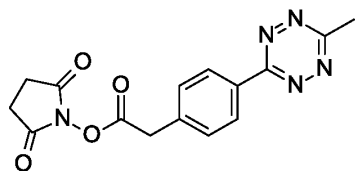
in the presence of a solvent and in the presence of a base to give Compound B.

15 In another embodiment, Compound B or a salt thereof, is obtained by combining

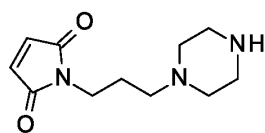
N,N'-disuccinimidyl carbonate, methyltetrazine-acid, ,
and a base in the presence of a solvent.

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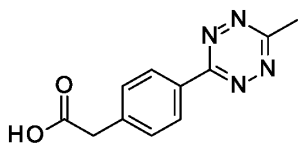
In another embodiment, a method of making Compound B or a salt thereof, comprises combining (2,5-dioxopyrrolidin-1-yl) 2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]acetate,



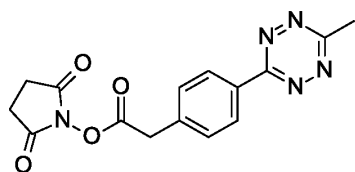
5 and 1-(3-piperazin-1-ylpropyl)pyrrole-2,5-dione,



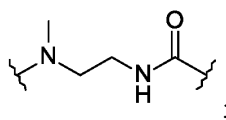
in the presence of a solvent and a base. In another embodiment, the method comprises combining N,N'-disuccinimidyl carbonate and a mixture of methyltetrazine-acid,



10 and a base in the presence of a solvent to give (2,5-dioxopyrrolidin-1-yl) 2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]acetate,



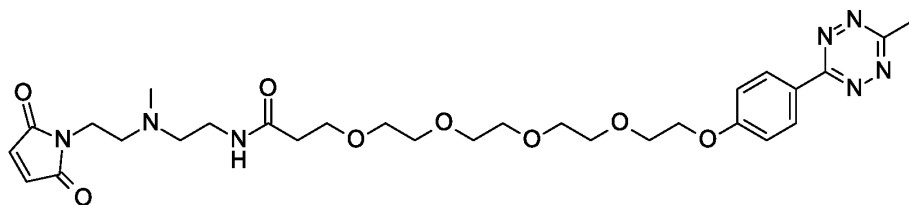
In an embodiment, R¹ is



15 or a salt thereof. In another embodiment, R² is PEG_n, wherein n is 1 to 10. In another embodiment, R² is PEG_n, wherein n is 2 to 6. In another embodiment, R³ is a -phenyl-tetrazine group, wherein the tetrazine is optionally substituted with methyl. In

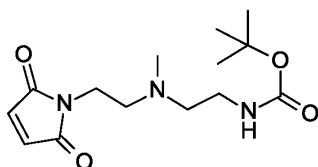
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another embodiment, the compound is

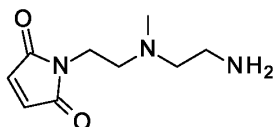


- 5 N-[2-[2-(2,5-dioxopyrrol-1-yl)ethyl-methyl-amino]ethyl]-3-[2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanamide (“Compound C”) or a salt thereof. In another embodiment, the compound is Compound C.

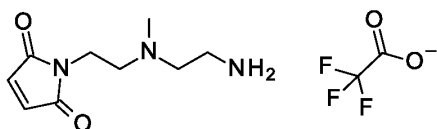
In another embodiment, Compound C, or a salt thereof, is obtained by combining tert-butyl N-[2-[2-(2,5-dioxopyrrol-1-yl)ethyl-methyl-amino]ethyl]carbamate,



- 10 and a deBoc reagent to give 1-[2-[2-aminoethyl(methyl)amino]ethyl]pyrrole-2,5-dione,



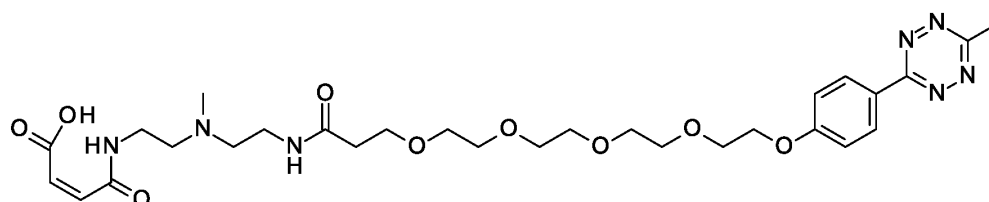
- In another embodiment, Compound C, or a salt thereof, is obtainable wherein the deBoc reagent comprises trifluoroacetic acid in DCM, HCl in dioxane, HCl in methanol, 15 or HCl in diethyl ether. In another embodiment, Compound C, or a salt thereof, is obtainable wherein the deBoc reagent comprises trifluoroacetic acid in DCM to give 1-[2-[2-aminoethyl(methyl)amino]ethyl]pyrrole-2,5-dione trifluoroacetate salt,



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In another embodiment, Compound C, or a salt thereof, is obtained by combining (Z)-4-[2-[methyl-2-[3-[2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy]ethoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanoylamino]ethyl]amino]ethylamino]-4-oxo-but-2-enoic acid,

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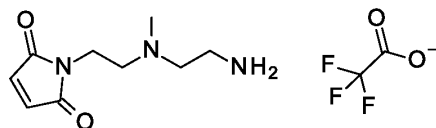


, acetate salt, and acetic anhydride. In another embodiment, Compound C, or a salt thereof, wherein acetate salt comprises sodium acetate.

In another embodiment, methods of making Compound C, or salts thereof are disclosed. In another embodiment, a method of making Compound C, or a salt thereof, comprises

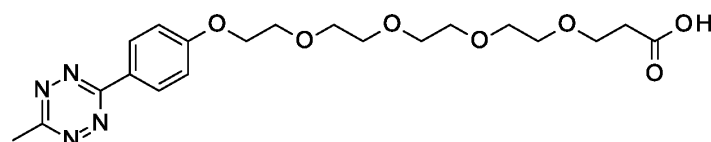
10

combining 1-[2-[2-aminoethyl(methyl)amino]ethyl]pyrrole-2,5-dione trifluoroacetate salt,

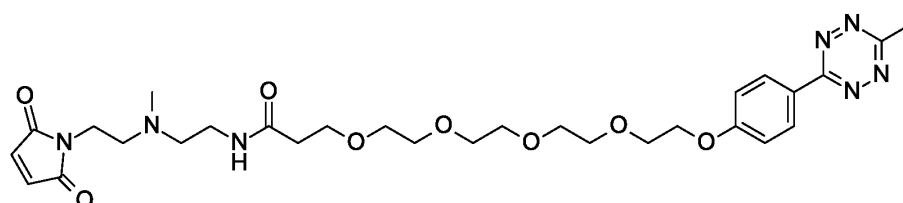


methyltetrazine-PEG4-acid,

15



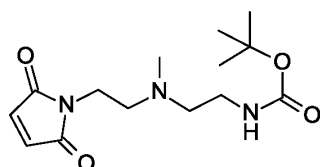
and an amide coupling reagent, and a base in a solvent to give N-[2-[2-(2,5-dioxopyrrol-1-yl)ethyl-methyl-amino]ethyl]-3-[2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanamide,



-28-

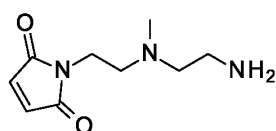
In another embodiment, a method of making Compound C, or a salt thereof, further comprising:

combining tert-Butyl N-[2-[2-(2,5-dioxopyrrol-1-yl)ethyl-methyl-amino]ethyl]carbamate,

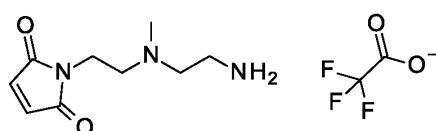


5

and a deBoc reagent to give 1-[2-[2-aminoethyl(methyl)amino]ethyl]pyrrole-2,5-dione,

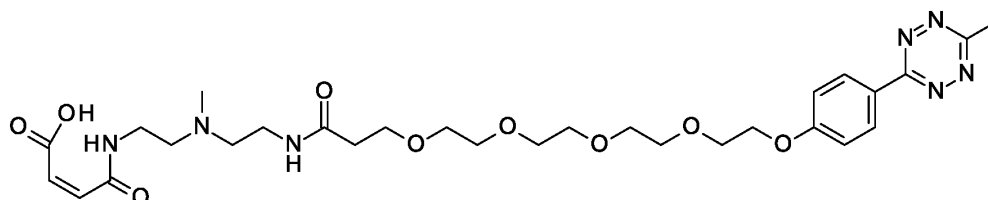


In another embodiment, a method of making Compound C, or a salt thereof, wherein the deBoc reagent comprises trifluoroacetic acid in DCM, HCl in dioxane, HCl in methanol, or HCl in diethyl ether. In another embodiment, a method of making Compound C, or a salt thereof, wherein the deBoc reagent comprises trifluoroacetic acid in DCM to give 1-[2-[2-aminoethyl(methyl)amino]ethyl]pyrrole-2,5-dione



trifluoroacetate salt,

In another embodiment, a method of making Compound C, or a salt thereof, combining (Z)-4-[2-[methyl-[2-[3-[2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy]ethoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanoylamino]ethyl]amino]ethylamino]-4-oxo-but-2-enoic acid,

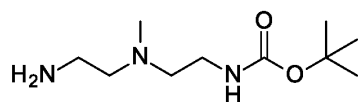


-29-

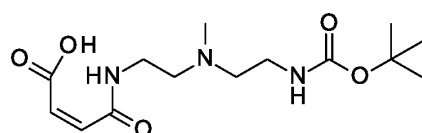
, acetate salt, and acetic anhydride. In another embodiment, a method of making Compound C, or a salt thereof, wherein acetate salt comprises sodium acetate.

In another embodiment, a method of making Compound C, or a salt thereof, further comprises the step of:

- 5 combining tert-butyl N-[2-[2-aminoethyl(methyl)amino]ethyl]carbamate,

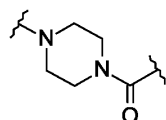


in acetic acid and a maleic agent to give the (Z)-4-[2-[2-(tert-butoxycarbonylamino)ethyl-methyl-amino]ethylamino]-4-oxo-but-2-enoic acid,



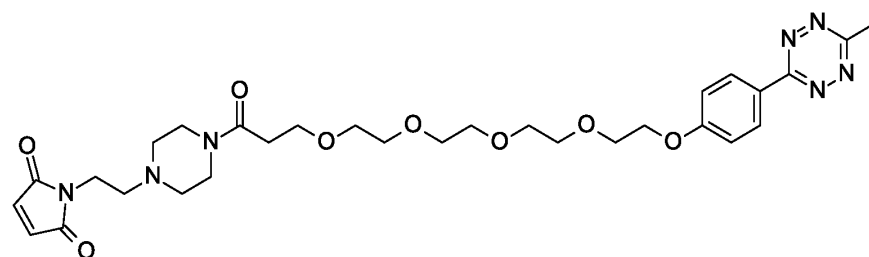
- 10 In another embodiment, a method of making Compound C, or a salt thereof, wherein the maleic agent is selected from the group consisting of maleic anhydride, maleic acid, maleamic acid, unsubstituted maleimide, and N-(methoxycarbonyl)maleimide.

In an embodiment, R¹ is



15

In another embodiment, R² is PEG_n, wherein n is 1 to 10. In another embodiment, R² is PEG_n, wherein n is 2 to 6. In another embodiment, R³ is a -phenyl-tetrazine group, wherein the tetrazine is optionally substituted with methyl. In another embodiment, the compound is

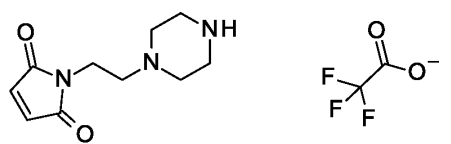


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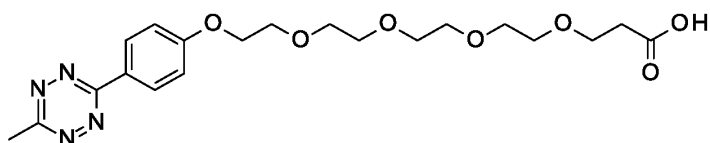
-30-

1-[2-[4-[3-[2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanoyl]piperazin-1-yl]ethyl]pyrrole-2,5-dione ("Compound D") or a salt thereof. In another embodiment, the compound is Compound D.

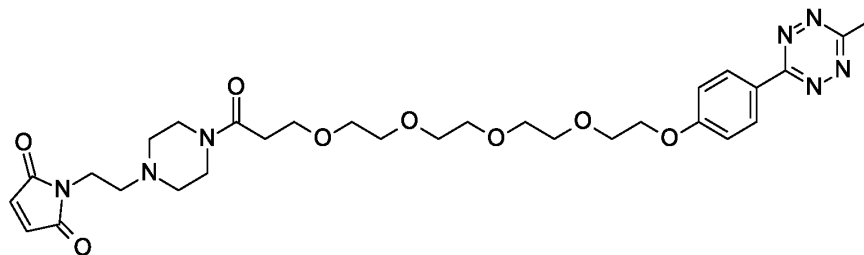
- 5 In another embodiment, Compound D, or a salt thereof, is obtained by combining 1-(2-piperazin-1-ylethyl)pyrrole-2,5-dione trifluoroacetate,



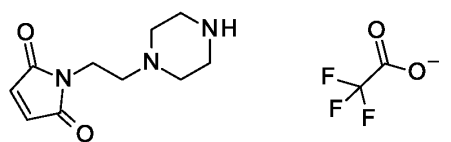
methyltetrazine-PEG4-acid,



- 10 an amide coupling reagent, and a base, to a solvent to give 1-[2-[4-[3-[2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanoyl]piperazin-1-yl]ethyl]pyrrole-2,5-dione,

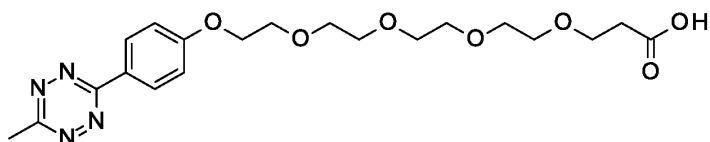


- 15 In another embodiment, Compound D, or a salt thereof, is obtained by combining 1-(2-piperazin-1-ylethyl)pyrrole-2,5-dione trifluoroacetate,

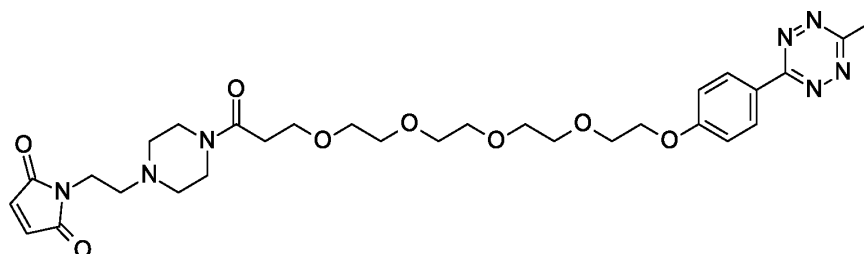


-31-

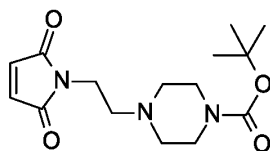
methyltetrazine-PEG4-acid,



an amide coupling reagent, and N,N-Diisopropylethylamine, to a solvent to give
 1-[2-[4-[3-[2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-
 5 yl)phenoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanoyl]piperazin-1-yl]ethyl]pyrrole-2,5-
 dione,

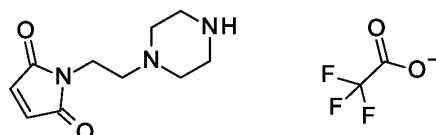


In another embodiment, Compound D, or a salt thereof, is obtained by combining
 tert-Butyl 4-[2-(2,5-dioxypyrrol-1-yl)ethyl]piperazine-1-carboxylate,



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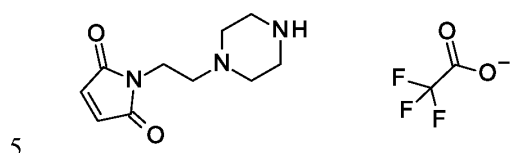
and a deBoc reagent in a solvent. In another embodiment, Compound D, or a salt
 thereof, wherein the deBoc reagent comprises trifluoroacetic acid in DCM, HCl in
 dioxane, HCl in methanol, or HCl in diethyl ether. In another embodiment, Compound D,
 or a salt thereof, wherein the deBoc reagent comprises trifluoroacetic acid in DCM to
 15 give 1-(2-piperazin-1-ylethyl)pyrrole-2,5-dione trifluoroacetate,



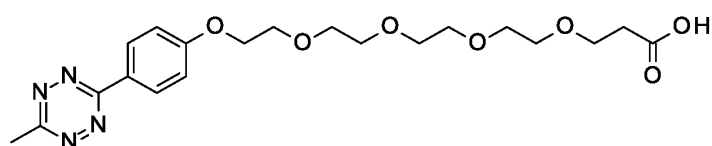
-32-

In another embodiment, methods of making Compound D, or salts thereof are disclosed. In another embodiment, a method of making Compound D, or a salt thereof, comprises:

combining 1-(2-piperazin-1-ylethyl)pyrrole-2,5-dione trifluoroacetate,

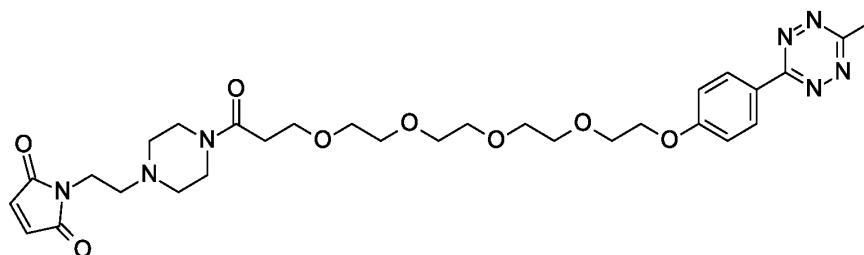


methyltetrazine-PEG4-acid,



an amide coupling reagent, and a base, to a solvent to give 1-[2-[4-[3-[2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy]ethoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanoyl]piperazin-1-yl]ethyl]pyrrole-2,5-dione,

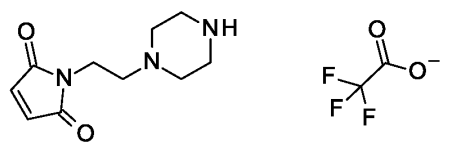
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In another embodiment, methods of making Compound D, or salts thereof are disclosed.

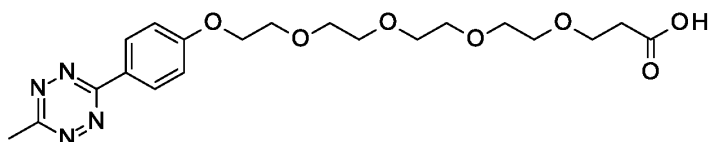
15 In another embodiment, a method of making Compound D, or a salt thereof, comprises:

combining 1-(2-piperazin-1-ylethyl)pyrrole-2,5-dione trifluoroacetate,

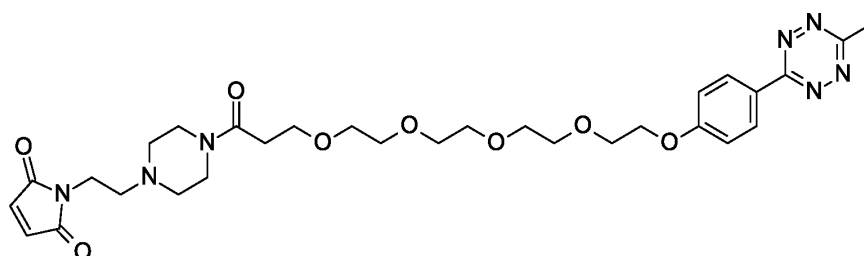


-33-

methyltetrazine-PEG4-acid,

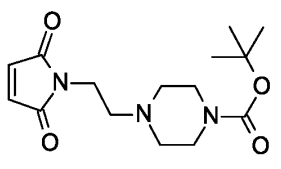


an amide coupling reagent, and N,N-Diisopropylethylamine, to a solvent to give
 1-[2-[4-[3-[2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-
 5 yl)phenoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanoyl]piperazin-1-yl]ethyl]pyrrole-2,5-
 dione,

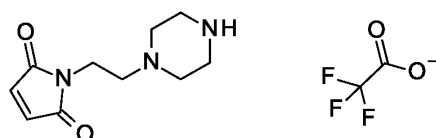


In another embodiment, a method of making Compound D, or a salt thereof,
 comprises:

10 combining tert-Butyl 4-[2-(2,5-dioxopyrrol-1-yl)ethyl]piperazine-1-carboxylate,



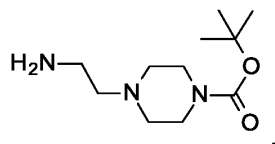
and a deBoc reagent in a solvent. In another embodiment, a method of making
 Compound D, or a salt thereof, wherein the deBoc reagent comprises trifluoroacetic acid
 in DCM, HCl in dioxane, HCl in methanol, or HCl in diethyl ether. In another
 15 embodiment, a method of making Compound D, or a salt thereof, wherein the deBoc
 reagent comprises trifluoroacetic acid in DCM to give 1-(2-piperazin-1-ylethyl)pyrrole-
 2,5-dione trifluoroacetate,



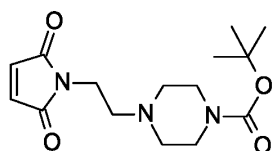
-34-

In another embodiment, a method of making Compound D, or a salt thereof, comprises:

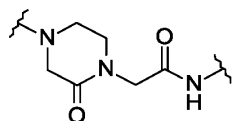
adding tert-butyl 4-(2-aminoethyl)piperazine-1-carboxylate,



5 furan-2,5-dione, acetic anhydride, and sodium acetate to a solvent to give tert-butyl 4-[2-(2,5-dioxopyrrol-1-yl)ethyl]piperazine-1-carboxylate,

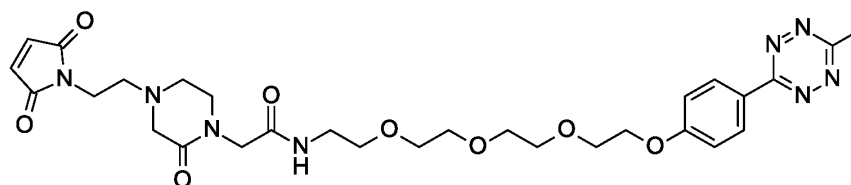


In an embodiment, R¹ is



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In another embodiment, R² is PEG_n, wherein n is 1 to 10. In another embodiment, R² is PEG_n, wherein n is 2 to 6. In another embodiment, R³ is a -phenyl-tetrazine group, wherein the tetrazine is optionally substituted with methyl. In another embodiment, the compound is

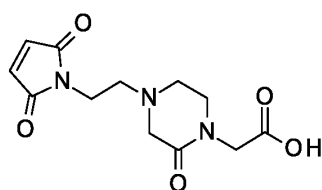


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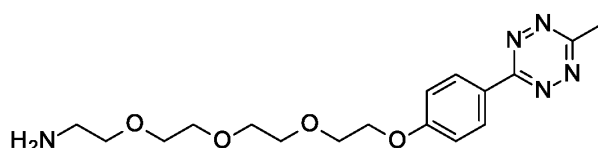
2-[4-[2-(2,5-dioxopyrrol-1-yl)ethyl]-2-oxopiperazin-1-yl]-N-[2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy]ethoxy]ethoxy]ethoxy]ethyl]acetamide (“Compound E”) or a salt thereof. In another embodiment, the compound is Compound E.

In another embodiment, Compound E, or a salt thereof, is obtained by combining
20 2-[4-[2-(2,5-Dioxopyrrol-1-yl)ethyl]-2-oxo-piperazin-1-yl]acetic acid,

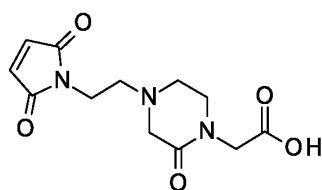
-35-



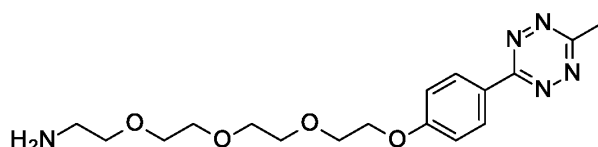
and methyltetrazine-PEG4-amine,



an amide coupling reagent, and an amide coupling solvent, and adding a base. In
 5 another embodiment, Compound E, or a salt thereof, is obtained by combining 2-[4-[2-(2,5-Dioxopyrrol-1-yl)ethyl]-2-oxo-piperazin-1-yl]acetic acid,



and methyltetrazine-PEG4-amine,

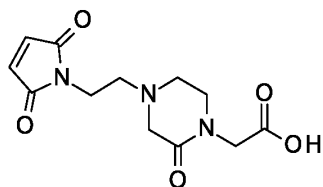


10 an amide coupling reagent, and an amide coupling solvent, and adding N,N-Diisopropylethylamine.

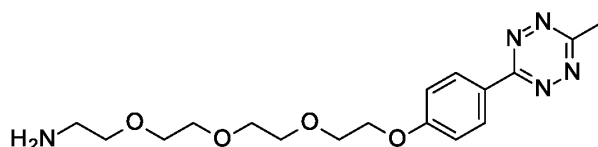
In another embodiment, methods of making Compound E, or salts thereof are disclosed. In another embodiment, a method of making Compound E, or a salt thereof, comprises:

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combining 2-[4-[2-(2,5-Dioxopyrrol-1-yl)ethyl]-2-oxo-piperazin-1-yl]acetic acid,



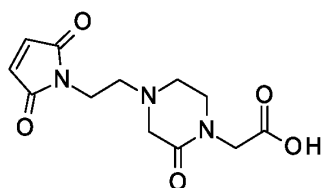
and methyltetrazine-PEG4-amine,



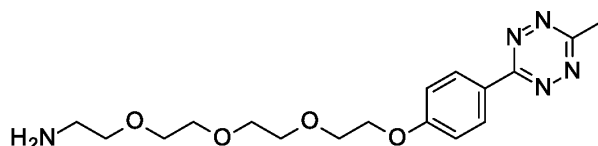
5 an amide coupling reagent, and an amide coupling solvent, and adding a base.

In another embodiment, a method of making Compound E, or a salt thereof, comprises:

combining 2-[4-[2-(2,5-Dioxopyrrol-1-yl)ethyl]-2-oxo-piperazin-1-yl]acetic acid,



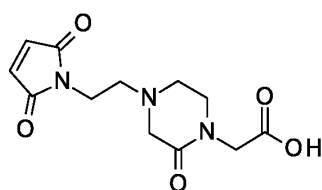
10 and methyltetrazine-PEG4-amine,



an amide coupling reagent, and an amide coupling solvent, and adding N,N-Diisopropylethylamine.

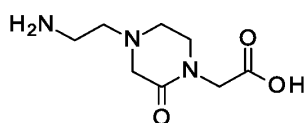
15 In another embodiment, when making Compound E, or a salt thereof, the 2-[4-[2-(2,5-dioxopyrrol-1-yl)ethyl]-2-oxo-piperazin-1-yl]acetic acid,

-37-

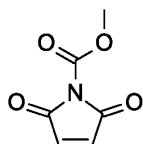


is prepared by a process comprising:

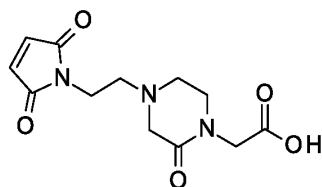
combining 2-[4-(2-aminoethyl)-2-oxopiperazin-1-yl]acetic acid,



5 and methyl 2,5-dioxo-2,5-dihydro-1H-pyrrole-1-carboxylate,

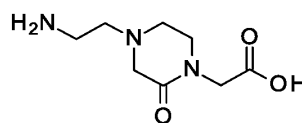


and a base in a solvent to give 2-[4-[2-(2,5-dioxopyrrol-1-yl)ethyl]-2-oxo-piperazin-1-yl]acetic acid,



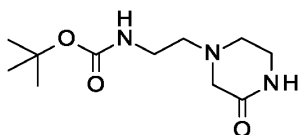
10 In another embodiment, when making Compound E, or a salt thereof, the 2-[4-(2-

aminoethyl)-2-oxopiperazin-1-yl]acetic acid,

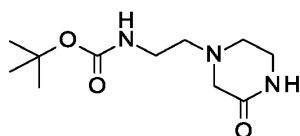


is prepared by a process comprising:

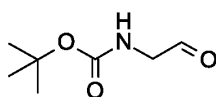
combining tert-Butyl N-[2-(3-oxopiperazin-1-yl)ethyl]carbamate,



sodium hydride, and tert-butyl bromoacetate to a solvent. In another embodiment, when making Compound E, or a salt thereof, the tert-butyl N-[2-(3-oxopiperazin-1-yl)ethyl]carbamate,



- 5 is prepared by a process comprising:
combining piperazin-2-one, tert-butyl N-(2-oxoethyl)carbamate,



acetic acid, and sodium triacetoxyborohydride.

- In all of the above embodiments, it is understood that a base is a substance that
10 reacts with an acid. In some embodiments, the base comprises a nitrogen containing base. In some embodiments, the nitrogen containing base comprises N,N-diisopropylethylamine, TEA, or pyridine. Examples of nitrogen containing bases include but are not limited to diisopropylethylamine, TEA, or pyridine. More preferred nitrogen containing base include N,N-diisopropylethylamine and TEA. A single base or mixtures
15 of two or more bases may be used.

Preferred embodiments of amide coupling solvent include DCM, DMF, DMA, or NMP. A single solvent or mixtures of two or more solvents may be used.

- In the above embodiments, a maleic agent is compound that comprises a dicarboxylic acid or an imide, wherein at least one of the carboxylic acids of the
20 dicarboxylic acid is optionally substituted with an amide. In some embodiments, the maleic agent comprises maleic anhydride, maleic acid, maleamic acid, unsubstituted maleimide, or N-(methoxycarbonyl)maleimide.

R3 binding

- The distal methyltetrazine of the exemplified molecules is designed to conjugate
25 to a biomolecule functionalized with trans-cyclooctene (TCO) through an inverse electron demand Diels-Alder (IEDDA) cycloaddition to form 4-linked 1-methyl-2,4a,5,6,7,8,9,10-octahydrocycloocta[d]pyridazine. There are various other ligation technologies that can also be employed on the distal end of R³. There are various other ligation technologies

that can also be employed on the distal end of R3. Examples of such ligation technologies are referred to herein as "ligation groups". Ligation groups that can be used include, but are not limited to:

1. Nitrogen containing ligation groups
 - Amides: coupling with N-hydroxysuccinimide or other activated esters, sometimes through amide coupling reagents
 - Amines: reductive amination with aldehyde/ketone
 - Imines: condensation with aldehyde/ketone.
 - Sulfonamides: coupling through sulfonyl fluorides
 - Thioureas/ureas: coupling with isothiocyanate/isocyanate
2. Sulfur containing (thiol typically from cysteine) ligation groups
 - Disulfide linkage: coupling with pyridyl sulfide, other activating groups
 - Aryl thioether: palladium-mediated coupling with aryl halide
 - Native chemical ligation (NCL): coupling C-terminal thioester with N-terminal cysteine
 - Thioether linkage:
 - Alpha-thioether carbonyl: coupling through alpha-halo carbonyl
 - Beta-thioether sulfone: coupling through mono-vinyl sulfone
 - Beta-thioether ester: coupling through acrylate
 - Thioether succinimide: coupled through maleimide
 - Alternative traps to thiolates include bis-vinyl sulfones, vinyl sulfonamides
3. Methods of making ligation groups
 - Cycloaddition:
 - [3+2] Copper-catalyzed alkyne/azide (Huisgen cycloaddition)
 - [3+2] Nitronc cycloaddition to olefins/alkynes
 - Strain-promoted alkyne/azide process:
 - Dibenzocyclooctyne (DBCO) coupled with azide to triazole product

- Tetrazine coupled with a TCO to octahydrocycloocta[d]pyridazine product

There are various modifying agents (R^3) to chemically modify a polypeptide, such as an antibody, to introduce novel active agents.

5 There are various drug payloads that can be bound to R_3 . Examples of such drug payloads include but are not limited to cytotoxin, immunostimulator, oligonucleotide, camptothecin analogs such as SN-38 and exatecan, maytansinoids such as maytansinoid DM1 and maytansinoid DM3, auristatins such as Monomethyl auristatin E (MMAE) and Monomethylauristatin F (MMAF), Tubulysins, DNA damaging agents such as PBD
10 dimers, and taxol derivatives such as docetaxel.

The present disclosure also relates to methods of making compounds of Formula I, and salts thereof.

The present disclosure also relates to methods of using compounds of Formula I, and salts thereof, for antibody-drug conjugation.

15 In an embodiment, triple phosphate buffer comprises the composition of 50 mM sodium phosphate monobasic monohydrate, 50 mM AMPPO, and 50mM $Na_4P_2O_7$.

The novel compounds comprising maleimide functional groups can be conjugated to a biological molecule, such as an antibody, a monoclonal antibody, or an antibody portion.

20 Suitable biological molecules include monoclonal antibody with engineered cysteines (or Engineered cysteine enabled mAb) comprising an IgG heavy chain and light chain constant region wherein the constant region comprises at least one cysteine. In an embodiment, the mAb constant region comprises at least one engineered cysteine at one of the following residues: residue 124 in the C_{H1} domain, residue 157 in the C_{H1} domain,
25 residue 162 in the C_{H1} domain, residue 262 in the C_{H2} domain, residue 375 in the C_{H3} domain, residue 373 in the C_{H3} domain, residue 397 in the C_{H3} domain, residue 415 in the C_{H3} domain, residue 156 in the C_{kappa} domain, residue 171 in the C_{kappa} domain, residue 191 in the C_{kappa} domain, residue 193 in the C_{kappa} domain, residue 202 in the C_{kappa} domain, or residue 208 in the C_{kappa} domain. See paragraphs [0085] to [0088] of US
30 Published patent application number 2020/0155702. In another embodiment, the constant region comprises at least one engineered cysteine at one of the following residues: residue 124 in the C_{H1} domain, residue 157 in the C_{H1} domain, residue 162 in the C_{H1} domain,

residue 262 in the C_{H2} domain, residue 375 in the C_{H3} domain, residue 373 in the C_{H3} domain, residue 378 in the C_{H3} domain, residue 397 in the C_{H3} domain, residue 415 in the C_{H3} domain, residue 156 in the C_{kappa} domain, residue 171 in the C_{kappa} domain, residue 191 in the C_{kappa} domain, residue 193 in the C_{kappa} domain, residue 202 in the C_{kappa} domain, or residue 208 in the C_{kappa} domain.

In another embodiment, the mAb comprises an IgG heavy chain constant region wherein the constant region comprises a cysteine at residue 124 in the C_{H1} domain, and a cysteine at one, but not all, of residue 157 and 162 in the C_{H1} domain and residues 375 and 378 in the C_{H3} domain. In another embodiment, the IgG heavy chain constant region is a human, mouse, rat or rabbit IgG constant region. In another embodiment, the IgG heavy chain constant region is a human IgG1, human IgG2, or human IgG4 isotype, and even more particularly, human IgG1 or human IgG4. In another embodiment, the IgG heavy chain constant region is a human IgG1 isotype. In another embodiment, mAb comprises human IgG1 heavy chain constant regions where the constant regions further comprise an isoleucine substituted at residue 247 and a glutamine substituted at residue 339. In another embodiment, the constant regions comprise an isoleucine substituted at residue 247, a glutamine substituted at residue 339, and a glutamic acid substituted at residue 332. In another embodiment, the IgG heavy chain constant region is a human IgG4 isotype. In another embodiment, mAb comprises human IgG4 heavy chain constant regions where the constant regions further comprise a proline substituted at residue 228, an alanine substituted at residue 234, and an alanine substituted at residue 235.

In another embodiment, the mAb comprises two heavy chain IgG constant regions wherein each IgG constant region comprises at least one cysteine. In another embodiment, each IgG constant region comprises a cysteine at one of the following residues: residue 124 in the C_{H1} domain, residue 157 in the C_{H1} domain, residue 162 in the C_{H1} domain, residue 375 in the C_{H3} domain, and residue 378 in the C_{H3} domain. In another embodiment, the mAb comprises two heavy chain IgG constant regions wherein each IgG constant region comprises a cysteine at residue 124 in the C_{H1} domain, and a cysteine at one, but not all, of residue 157 and 162 in the C_{H1} domain and residues 375 and 378 in the C_{H3} domain of each heavy chain. In another embodiment, each IgG constant region is human, mouse, rat or rabbit IgG. In another embodiment, each IgG

constant region is human IgG1, human IgG2, or human IgG4 isotype. In another embodiment, each IgG constant region is human IgG1 or human IgG4. In another embodiment, each IgG heavy chain constant region is a human IgG1 isotype. In another embodiment, two human IgG1 heavy chain constant regions further comprise an
5 isoleucine substituted at residue 247 and a glutamine substituted at residue 339. In another embodiment, the constant regions comprise an isoleucine substituted at residue 247, a glutamine substituted at residue 339, and a glutamic acid substituted at residue 332. In another embodiment, each IgG heavy chain constant region is a human IgG4 isotype. In another embodiment, two human IgG4 heavy chain constant regions further
10 comprise a proline substituted at residue 228, an alanine substituted at residue 234, and an alanine substituted at residue 235.

Unless otherwise expressly stated herein, all references to residues appearing in the specification and Examples are based on the EU Index Numbering system. See paragraph [0098] of US Published patent application number 2020/0155702.

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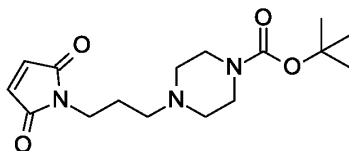
Preparation 0

Triple phosphate buffer composition

13.8 grams of sodium phosphate monobasic monohydrate, 22.7 grams of APMSO and 26.6 grams of Na₄P₂O₇ were added to 2L flask and solubilized using de-ionized
20 filtered water in 2L. This 2L solution was subdivided into 250mL buffer solution to generate varied pH solution ranging from 5 to 8 by adjusting the pH using either 1M sodium hydrochloride stock or 1N sodium hydroxide.

Preparation 1

tert-butyl 4-[3-(2,5-dioxopyrrol-1-yl)propyl]piperazine-1-carboxylate

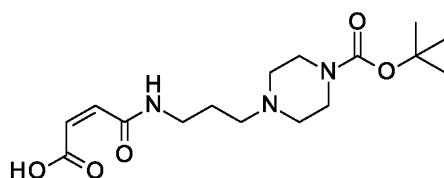


25

Maleic anhydride (789 mg, 7.97 mmol) was added to a solution of tert-butyl 4-(3-aminopropyl)piperazine-1-carboxylate (2.00 g, 7.97 mmol) in acetic acid (8 mL, 140 mmol). The mixture was stirred at rt for 12 hours. A faint yellow solution was obtained. The volatiles were removed under reduced pressure to a residue that was dried

-43-

under vacuum. The crude intermediate (Z)-4-[3-(4-tert-butoxycarbonylpiperazin-1-yl)propylamino]-4-oxo-but-2-enoic acid,

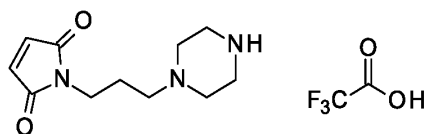


, ("Preparation 1 Intermediate 1")

(2.72 g, 7.97 mmol) was dissolved in toluene (80 mL, 750 mmol). Triethylamine (5.6 mL, 40 mmol) and 4Å molecular sieves (8.8 g) were added. The flask was equipped with a Dean-Stark trap, and the mixture was heated at 120 °C for 48 hours. After cooling to rt, the solids were removed by filtration, washing the solids with DCM (40 mL). The volatiles were removed under reduced pressure to a residue that was dried under vacuum. The thick residue was purified by normal phase chromatography ([10% MeOH/MTBE]/DCM). The title compound was isolated (353 mg, 1.09 mmol, 13.7% yield) as a yellow, flaky powder. MS m/z 324 (M+1).

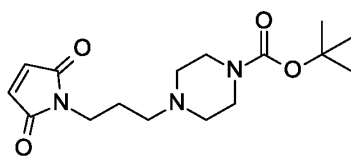
Preparation 2

1-(3-piperazin-1-ylpropyl)pyrrole-2,5-dione, trifluoroacetic acid



15

Trifluoroacetic acid (2.0 mL, 26 mmol) was added dropwise to an ice-cold solution of tert-butyl 4-[3-(2,5-dioxopyrrol-1-yl)propyl]piperazine-1-carboxylate,



, ("Preparation 1")

(353 mg, 1.09 mmol) in DCM (5.0 mL). A clear solution was obtained. The mixture was stirred for 2 h, and the volatiles were removed under reduced pressure to a residue that was dried under vacuum. The title compound was isolated (325 mg, 0.964 mmol, 88.4% yield) as a white powder. MS m/z 224 (M+1).

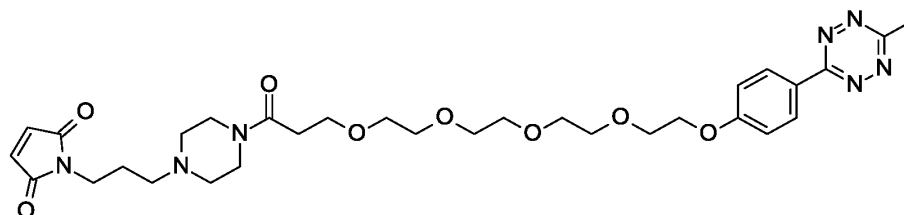
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-44-

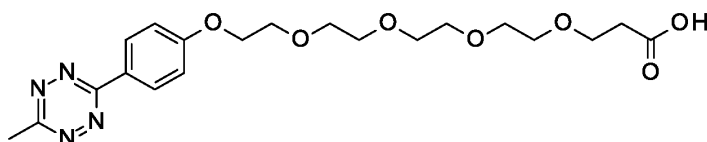
Example 1

1-[3-[4-[3-[2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy]ethoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanoyl]piperazin-1-yl]propyl]pyrrole-2,5-dione ("Compound A")

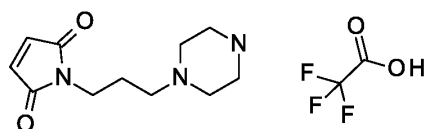
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Under nitrogen atmosphere, HATU (85 mg, 0.22 mmol) was added to a solution of methyltetrazine-PEG4-acid (50 mg, 0.11 mmol),



10 in DMF (0.5 mL), and the resulting purple activated acid mixture was stirred for 10 min at rt. In a separate vessel, 1-(3-piperazin-1-ylpropyl)pyrrole-2,5-dione trifluoroacetic acid (27 mg, 0.080 mmol),



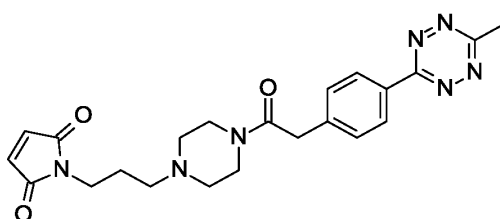
and N,N-diisopropylethylamine (60 μ L, 0.34 mmol) in DMF (0.22 mL) were
 15 mixed at rt. After 10 min., this mixture was added to the activated acid mixture via syringe. A purple solution was obtained. After 90 min, an additional 1-(3-piperazin-1-ylpropyl)pyrrole-2,5-dione (27 mg, 0.12 mmol) was added, and the mixture was stirred at rt for an additional 90 min, and then placed in a -20 $^{\circ}$ C freezer for storage overnight. The reaction was diluted with water (20 mL) and DCM (40 mL). The layers were separated,
 20 and the organic layer was washed with water (2 $\times$ 20 mL), then satd aq ammonium chloride, satd aq sodium bicarbonate, and then with satd aq sodium chloride. The organics were dried over magnesium sulfate, filtered, and concentrated to dryness to give a purple

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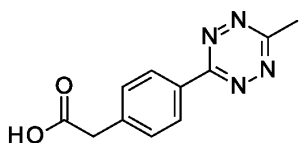
residue. Isolated title compound (42.3 mg, 0.0659 mmol, 60.6% yield) as a purple residue. MS m/z 642 ($M+1$).

Example 2

1-[3-[4-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]acetyl]piperazin-1-yl]propyl]pyrrole-2,5-dione ("Compound B")



Under N_2 atmosphere, N,N' -disuccinimidyl carbonate (61 mg, 0.23 mmol) was added to a mixture of methyltetrazine-acid,



10

(50 mg, 0.21 mmol), N,N -diisopropylethylamine (75 μ L, 0.43 mmol) in dichloromethane (4.1 mL). The purple mixture was stirred at rt for 12 h. The purple reaction solution was diluted with water (10 mL) and the aqueous layer was extracted with DCM (2×10 mL). The combined organic layers were washed with satd aq ammonium chloride, satd aq sodium bicarbonate, then with brine. The organics were dried over magnesium sulfate, filtered, and concentrated to dryness. (2,5-dioxopyrrolidin-1-yl) 2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]acetate (99.7 mg, 0.305 mmol) was isolated as a purple solid. This material was dissolved in DCM (3.0 mL). Under N_2 atmosphere, this solution was slowly added to a solution of 1-(3-piperazin-1-ylpropyl)pyrrole-2,5-dione (49 mg, 0.22 mmol) and N,N -diisopropylethylamine (90 μ L, 0.51 mmol) in THF (1.0 mL) and dichloromethane (3.0 mL). A purple solution was obtained. After 2 min, additional 1-(3-piperazin-1-ylpropyl)pyrrole-2,5-dione (49 mg, 0.22 mmol) as a solid was added, and the mixture was stirred at rt for an additional 90 min. The volatiles were removed under reduced pressure to a residue that was absorbed onto silica gel (1 g) with the aid of DCM (10 mL). The volatiles were removed

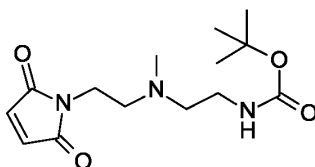
-46-

under reduced pressure to a free flowing residue that was purified by chromatography (12 g silica gel; 3 min DCM, then 6 min 5% [10% MeOH/MTBE]/DCM, then 5 min 10% [10% MeOH/MTBE]/DCM). The title compound was isolated (48 mg, 50% yield) as a purple foam. MS m/z 436 (M+1).

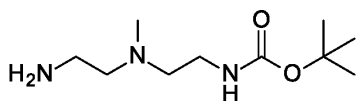
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Preparation 3

tert-butyl N-[2-[2-(2,5-dioxopyrrol-1-yl)ethyl-methyl-amino]ethyl]carbamate



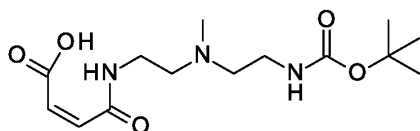
Maleic anhydride (564 mg, 5.75 mmol) was added to a solution of tert-butyl N-[2-[2-aminoethyl(methyl)amino]ethyl]carbamate,



10

(1.25 g, 5.75 mmol) in acetic acid (4 mL, 140 mmol). The mixture was stirred at rt for 7 h. A faint yellow solution was obtained. The volatiles were removed under reduced pressure to a residue that was dried under vacuum for a weekend. The crude intermediate, (Z)-4-[2-[2-(tert-butoxycarbonylamino)ethyl-methyl-amino]ethylamino]-4-oxo-but-2-enoic acid,

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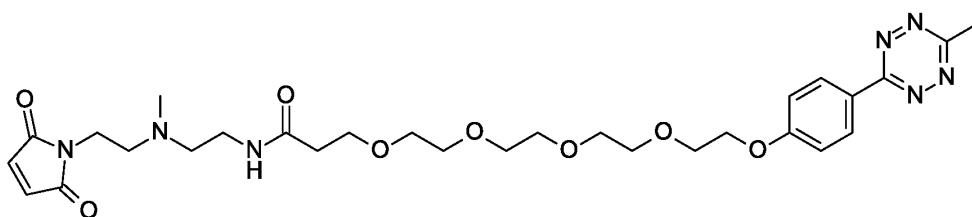
(1.81 g, 5.75 mmol), was dissolved in toluene (30 mL). Triethylamine (4 mL, 29 mmol) and 4Å molecular sieves (3.0 g) were added. The mixture was heated to refluxing for 6 h. After cooling to rt, the mixture was filtered through a diatomaceous earth pad, washing the pad with DCM (3 × 5 mL). The filtrate was concentrated under vacuum to give a thick residue. The residue was dissolved in DCM (50 mL) and washed with water (20 mL). The organic layer was separated, dried over anhydrous sodium sulfate, filtered, and concentrated under vacuum. The resulting residue was purified by

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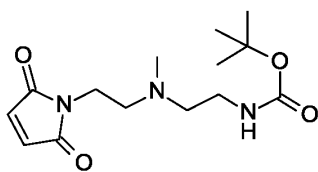
normal phase chromatography (EtOAc/hexane). The title compound was isolated (580 mg, 1.95 mmol, 33.9% yield) as a pale yellow semi-solid. MS m/z 298 (M+1).

Example 3

N-[2-[2-(2,5-dioxopyrrol-1-yl)ethyl-methyl-amino]ethyl]-3-[2-[2-[2-[2-[4-(6-methyl-
5 1,2,4,5-tetrazin-3-yl)phenoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanamide ("Compound C")

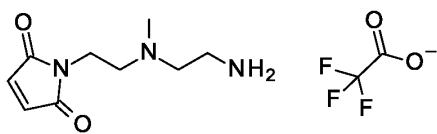


tert-Butyl N-[2-[2-(2,5-dioxopyrrol-1-yl)ethyl-methyl-amino]ethyl]carbamate,



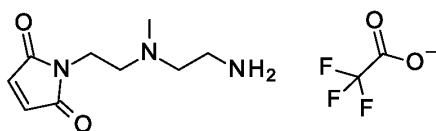
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(75 mg, 0.25 mmol) was dissolved in trifluoroacetic acid (2.0 mL, 26 mmol) and DCM (4 mL) and stirred at rt for 1 h. The volatiles were removed under reduced pressure to a residue that was dried under vacuum for overnight to give 1-[2-[2-aminoethyl(methyl)amino]ethyl]pyrrole-2,5-dione trifluoroacetate salt,



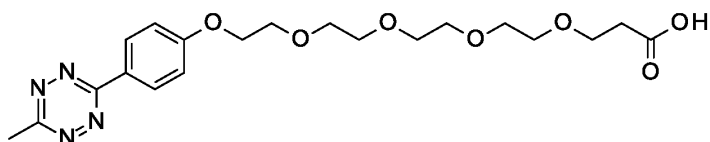
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as white solid. 1-[2-[2-aminoethyl(methyl)amino]ethyl]pyrrole-2,5-dione trifluoroacetate salt,

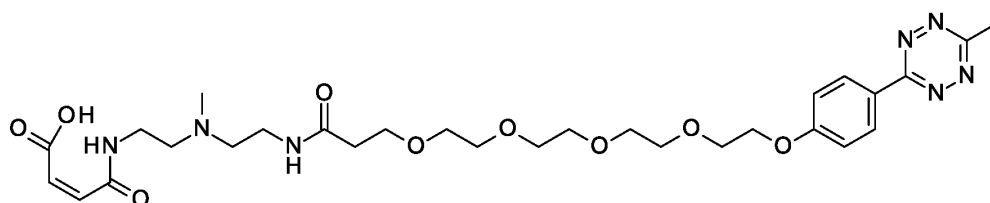


-48-

methyltetrazine-PEG4-acid,



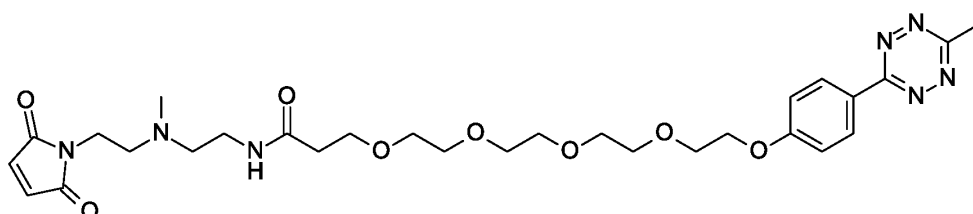
(100 mg, 0.22 mmol), and HATU (200 mg, 0.51 mmol) were mixed together in DMF (2.0 mL) and THF (1.0 mL). To the solution, N,N-diisopropylethylamine (170 μ L, 1.01 mmol) was added via a syringe. The mixture was stirred at rt for 2 h. The reaction was then diluted with DCM (50 mL) and washed with saturated aqueous ammonium chloride. The organic layer was separated, dried over sodium sulfate, filtered, and concentrated to dryness to a dark red oil. Purified by normal phase flash column chromatography (MeOH/DCM). Early fractions yielded the title compound (74 mg, 0.25 mmol, 40.5% yield) as a red solid. MS m/z 616.2 (M+1). Later fractions yielded a ring-opened product, (Z)-4-[2-[methyl-[2-[3-[2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanoylamino]ethyl]amino]ethylamino]-4-oxo-but-2-enoic acid,



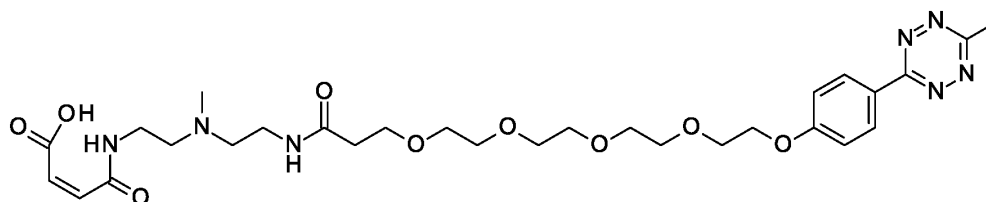
, as purple solid (65 mg, yield 26%). MS m/z 634 (M+1).

Example 3A

N-[2-[2-(2,5-dioxopyrrol-1-yl)ethyl-methyl-amino]ethyl]-3-[2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanamide ("Compound C")



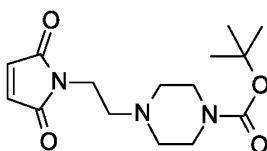
(Z)-4-[2-[methyl-[2-[3-[2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanoylamino]ethyl]amino]ethylamino]-4-oxo-but-2-enoic acid (105 mg, 0.166 mmol),



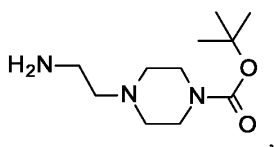
- 5 was dissolved in acetic anhydride (2 mL). Sodium acetate (50 mg, 0.61 mmol) was then added. The mixture was heated at 70 °C for 4 h. The acetic anhydride was removed under reduced pressure. The residue was dissolved in DCM (50 mL) and washed with water (20 mL). The organic phase was separated, dried over anhydrous sodium sulfate, filtered, and concentrated under vacuum. The resulting residue was purified by RP-HPLC (C18
10 column, A: 5% ammonium carbonate in water, B: ACN, 5-32% B over 6.5 min). The title compound was obtained as purple solid (30 mg, 28% yield). MS m/z 616.6 (M+1).

Preparation 4

tert-butyl 4-[2-(2,5-dioxopyrrol-1-yl)ethyl]piperazine-1-carboxylate



- 15 tert-Butyl 4-(2-aminoethyl)piperazine-1-carboxylate,



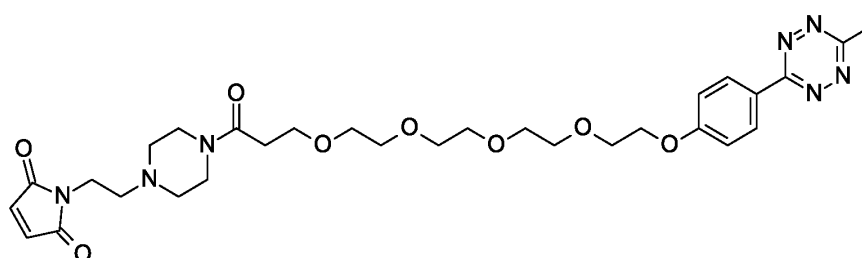
- (3.00 g, 13.1 mmol) was dissolved in acetic acid (6 mL). Added furan-2,5-dione (1.28 g, 13.1 mmol). Stirred at rt for 7 h. The mixture was then stored in a refrigerator for overnight. Most of acetic acid was removed under vacuum (50 °C). Added acetic
20 anhydride (10 mL, 106 mmol) and sodium acetate (1.6 g, 20 mmol). Heated to 80 °C for 2 h. Most of the acetic anhydride was removed under vacuum (with toluene). The mixture was taken into satd aq ammonium chloride (60 mL) giving pH 5 and extracted with DCM (3 × 50 mL). The combined organic layers were dried over anhydrous sodium sulfate,

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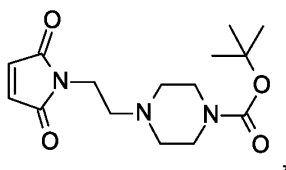
filtered, and concentrated under vacuum to give crude product as a dark oil. This material was purified with flash column chromatography (80 g silica gel, EtOAc/hexane). The title compound was isolated (2.1 g, 6.8 mmol, 52% yield). MS m/z 310.3 (M+1).

Example 4

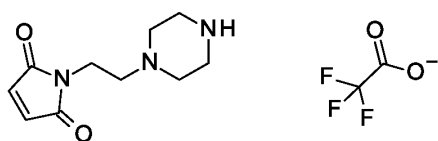
- 5 1-[2-[4-[3-[2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanoyl]piperazin-1-yl]ethyl]pyrrole-2,5-dione ("Compound D")



- 10 tert-Butyl 4-[2-(2,5-dioxopyrrol-1-yl)ethyl]piperazine-1-carboxylate,

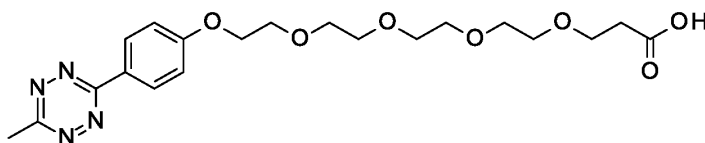


- (150 mg, 0.485 mmol) was dissolved in DCM (2 mL). Then added trifluoroacetic acid (1 mL, 13 mmol) and stirred at rt for 1 h. Solvent was removed under vacuum to dryness and further dried under high vacuum overnight to give intermediate 1-(2-piperazin-1-ylethyl)pyrrole-2,5-dione trifluoroacetate,
- 15



1-(2-piperazin-1-ylethyl)pyrrole-2,5-dione trifluoroacetate and methyltetrazine-PEG4-acid,

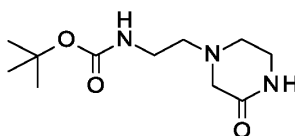
-51-



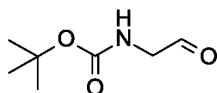
(130 mg, 0.283 mmol) were dissolved in DMF (2.0 mL) and THF (2 mL). HATU (380 mg, 0.969 mmol) was then added followed by N,N-diisopropylethylamine (0.45 mL, 2.6 mmol). Stirred at rt for 2 h. Diluted with DCM (50 mL). Washed with satd aq ammonium chloride (30 mL). The aqueous phase was pH 6. The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated under vacuum to give crude product as a red solid. Purified by normal phase flash chromatography (12 g silica gel, 0-20% MeOH/EtOAc over 15 min). The title compound was isolated (150 mg, 0.239 mmol, 49% yield) as a red solid. MS m/z 628.6 (M+1).

Preparation 5

tert-butyl N-[2-(3-oxopiperazin-1-yl)ethyl]carbamate



Piperazin-2-one (510 mg, 5.10 mmol) and tert-butyl N-(2-oxoethyl)carbamate,

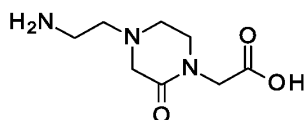


(0.91 g, 5.7 mmol) were mixed together in THF (20 mL) to form a clear solution. Added acetic acid (0.6 mL, 10 mmol) and stirred at rt for 5 min. Sodium triacetoxyborohydride (3.33 g, 15.2 mmol) was added and stirred at rt under N₂ for 4 h. The mixture was then diluted with EtOAc (100 mL) and washed with brine and water. The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated under vacuum. The resulting residue was purified by normal phase chromatography (0-50% MeOH/DCM). The title compound was isolated (1.0 g, 4.1 mmol, 81% yield) as a pale yellow solid. MS m/z 243.9 (M+1).

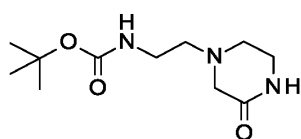
-52-

Preparation 6

2-[4-(2-aminoethyl)-2-oxopiperazin-1-yl]acetic acid



tert-Butyl N-[2-(3-oxopiperazin-1-yl)ethyl]carbamate,



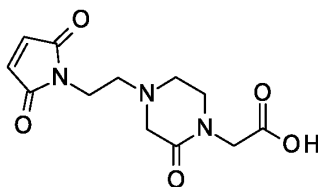
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(Preparation 5, 350 mg, 1.44 mmol) was dissolved in THF (3.0 mL). Sodium hydride (60 mass% in mineral oil) (74 mg, 1.9 mmol) was added. The reaction was stirred at rt under N₂ for 20 min. A solution of tert-butyl bromoacetate (364 mg, 1.87 mmol) in THF (0.5 mL) was then added via syringe and continued stirring for 3 h. The mixture was then diluted with water (50 mL) and extracted with EtOAc (3 × 30 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered, and concentrated under vacuum to give crude intermediate. The crude was then dissolved in trifluoroacetic acid (3 mL, with 30 µL of triisopropylsilane) and stirred at rt for 30 min. Most of the trifluoroacetic acid was removed under vacuum. The residue was dissolved in methanol and passed through an SCX column (10 g), eluting with 2 M ammonia in methanol. The title compound was isolated as dark solid (180 mg, 0.89 mmol, 62% yield). MS m/z 202 (M+1).

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Preparation 7

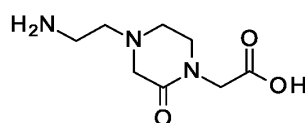
2-[4-[2-(2,5-dioxopyrrol-1-yl)ethyl]-2-oxo-piperazin-1-yl]acetic acid



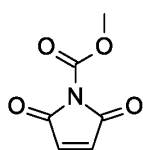
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2-[4-(2-Aminoethyl)-2-oxopiperazin-1-yl]acetic acid,

-53-



(180 mg, 0.89 mmol) was dissolved in 1 M aq sodium bicarbonate (3 mL, 3 mmol) and cooled in an ice-water bath. Methyl 2,5-dioxo-2,5-dihydro-1H-pyrrole-1-carboxylate,

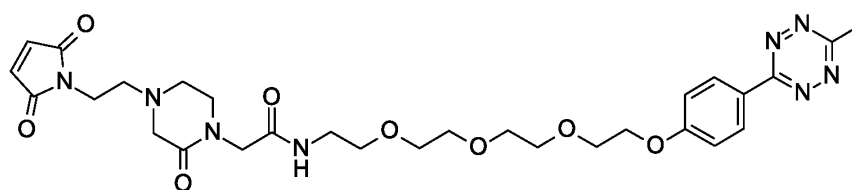


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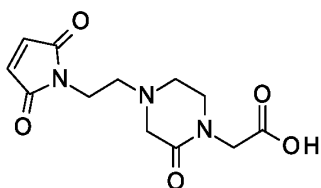
(128 mg, 0.80 mmol) was added to the solution and stirred at 0 °C for 1 h. The mixture was then diluted with water (10 mL) and washed with 3:1 chloroform:IPA (3 × 20 mL). The aqueous layer was adjusted to about pH 6 with 5 M aq HCl and extracted with 3:1 chloroform:IPA (3 × 20 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered, and concentrated under vacuum to give title compound (35 mg, 0.12 mmol, 14% yield). MS m/z 282 (M+1). The aqueous phase was then frozen and lyophilized. This gave crude title compound (200 mg, 0.36 mmol, 39%). MS m/z 282 (M+1).

Example 5

2-[4-[2-(2,5-dioxopyrrol-1-yl)ethyl]-2-oxopiperazin-1-yl]-N-[2-[2-[2-[2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy]ethoxy]ethoxy]ethoxy]ethyl]acetamide ("Compound E")



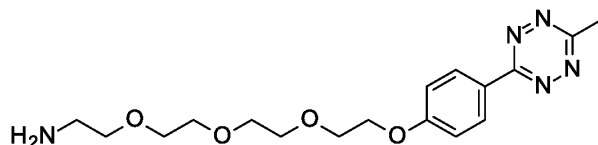
2-[4-[2-(2,5-Dioxopyrrol-1-yl)ethyl]-2-oxo-piperazin-1-yl]acetic acid,



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-54-

(80 mg, 0.14 mmol), methyltetrazine-PEG4-amine,



(35 mg, 0.092 mmol), and HATU (71 mg, 0.18 mmol) were dissolved in DMF (1.0 mL, 13 mmol) and THF (1 mL) and stirred at rt for 5 min. N,N-diisopropylethylamine (80 μ L, 0.46 mmol) was then added to the solution and stirred at rt for 1 h. The mixture was then diluted with water (50 mL) and extracted with 3:1 chloroform:IPA (2×20 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered, and concentrated under vacuum to give dark red oil. Purified twice by normal phase flash chromatography (0-20% methanol/DCM). Isolated Compound E (10 mg, 0.015 mmol, 17% yield). MS m/z 627.5 (M+1).

Example 6

Self-hydrolysis assessment of linkers

Self-hydrolysis of the linkers were assessed by incubating the linkers in triple phosphate buffer with corresponding pH 6.0, 6.5, 7.0, 7.5 and 8.0. Lyophilized linker powders were solubilized in 100% DMSO to make up 80 mM stock solution. Each of the linkers were incubated in the buffer at final linker concentration of 1.5 mM to determine succinimide hydrolysis at room temperature for 1 hour. Effect of pH on linker self-hydrolysis was assessed at 0.6, 4, and 20 hour intervals by Reverse Phase-HPLC mass-spec analysis. Table 4 provides hydrolysis percentages at 0.6, 4, and 20 hour intervals. "nd" is no data.

Scheme 1: Maleimide self-hydrolysis

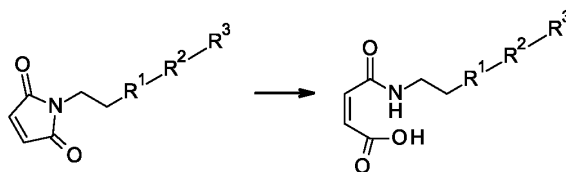
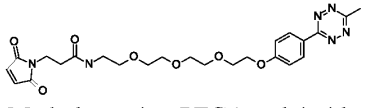
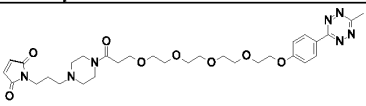
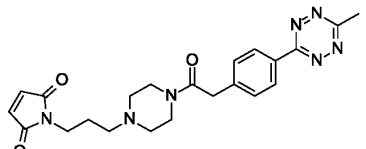
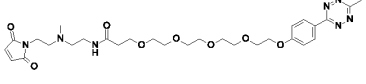
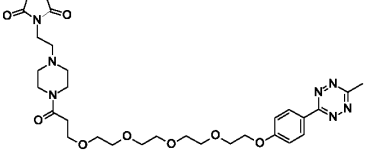
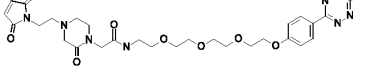


Table 4: Maleimide self-hydrolysis

Example	Precursor Structure	pH	0.6 h	4 h	20 h
commercial		6.0	0.56	0.80	2.00
		6.5	0.58	0.40	4.90
		7.0	1.26	1.70	12.30
		7.5	3.12	3.70	29.10

	 Methyltetrazine-PEG4-maleimide	8.0	8.47	10.20	83.70
1 (Compound A)		6.0	5.60	57.00	68.50
		6.5	11.99	87.70	93.40
		7.0	16.55	98.10	94.90
		7.5	25.82	100.00	99.10
		8.0	39.04	100.00	99.70
2 (Compound B)		6.0	9.96	18.90	72.80
		6.5	19.04	38.20	94.90
		7.0	32.69	59.20	99.40
		7.5	45.44	75.50	100.00
		8.0	58.67	90.10	100.00
3 (Compound C)		6.0	80.66	95.10	nd
		6.5	97.37	95.60	nd
		7.0	98.01	95.80	nd
		7.5	98.13	95.30	nd
		8.0	98.14	95.90	nd
4 (Compound D)		6.0	88.80	92.50	92.50
		6.5	92.90	92.80	93.10
		7.0	94.10	93.10	93.40
		7.5	94.50	93.60	93.40
		8.0	94.60	93.60	93.50
5 (Compound E)		6.0	9.70	22.30	59.70
		6.5	16.50	37.70	79.30
		7.0	32.10	63.30	93.20
		7.5	51.70	82.20	96.30
		8.0	73.20	93.60	95.80

Example 7

Conjugation assessment of linkers

Monoclonal antibody (mAb) with site-specific engineered cysteine was used to assess conjugation of these linkers via maleimide thiol-chemistry reaction. mAbs were diluted to 10 mg/mL into the aforementioned phosphate buffer in pH 6.0, 6.5, 7.0, 7.5, and 8.0. Each of the linkers were added at 20 molar equivalents to the mAbs and incubated for 1 hour at room temperature to conjugate. Following 1 hour incubation, excess linker was removed by desalting the solution back to buffer in pH 6.0, 6.5, 7.0, 7.5, and 8.0 by spin desalting columns using standard manufacturer protocol. Effect of pH on linker

conjugation to the mAb and post-conjugation hydrolysis of the linkers were assessed over time by Reverse Phase-LCMS.

Reverse Phase-HPLC mass-spec analysis was used to assess conjugation of the linker to mAb and post-conjugation hydrolysis of the linkers. For this analysis, 10 μ L 1mg/mL of antibody-linker conjugates were prepared. Partial reduction was performed by adding 1.5 μ L 100 mM TCEP and 1 μ L of 1M Tris-HCl pH 8.0 to each sample and incubating at 37 $^{\circ}$ C for 30 minutes. Antibody-linker conjugates were analyzed at Day 0, Day 3. Samples were pH adjusted by 1 M Tris-HCl to pH 8.0.

Table 5 provides conjugation rate percentages and post-conjugation hydrolysis rate percentages at day 0 and day 3 intervals.

Scheme 2: Maleimide conjugation followed by self-hydrolysis

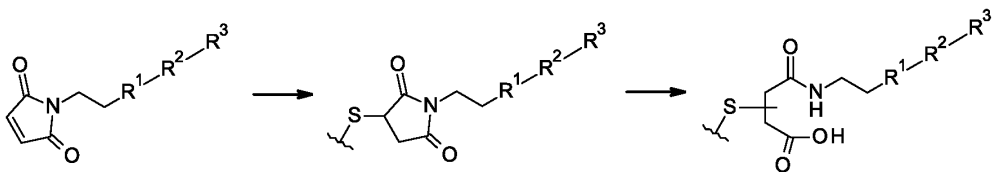
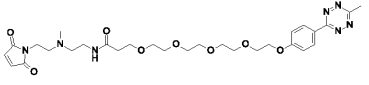
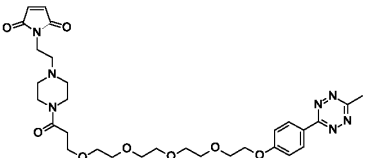
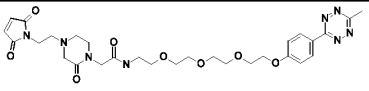


Table 5: Maleimide conjugation rate and post-conjugation self-hydrolysis rate

Example	Precursor Structure	pH	Conjugation rate		Post-conjugation hydrolysis rate	
			Day 0	Day 3	Day 0	Day 3
commercial		6.0	53	53	6	5
		6.5	77	77	7	6
		7.0	92	93	8	9
		7.5	95	94	8	17
		8.0	92	92	13	44
1 (Compound A)		6.0	97	97	10	9
		6.5	97	98	10	20
		7.0	96	97	13	42
		7.5	95	95	15	69
		8.0	92	93	20	85
2 (Compound B)		6.0	96	99	9	10
		6.5	97	97	10	23
		7.0	95	96	12	44
		7.5	93	94	18	68
		8.0	88	91	23	85
		6.0	96	99	94	92
		6.5	97	98	95	96

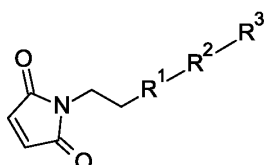
3 (Compound C)		7.0	96	97	95	97
		7.5	95	96	93	96
		8.0	92	94	92	96
4 (Compound D)		6.0	51	65	27	35
		6.5	65	68	27	46
		7.0	67	67	29	65
		7.5	66	66	34	80
		8.0	66	61	41	100
5 (Compound E)		6.0	78	78	31	86
		6.5	77	77	68	84
		7.0	76	78	71	85
		7.5	77	78	74	85
		8.0	74	77	77	85

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CLAIMS

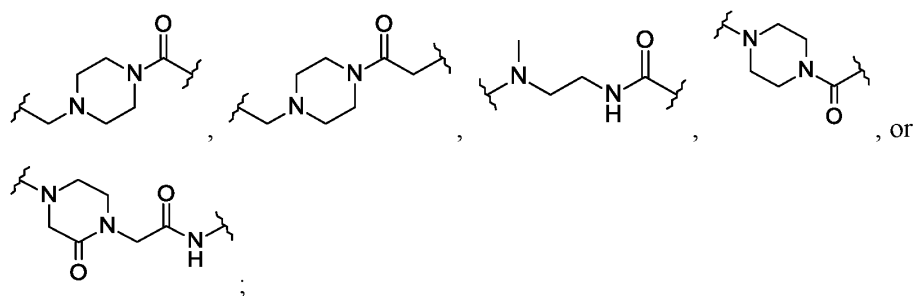
WE (I) CLAIM:

1. A compound of formula:

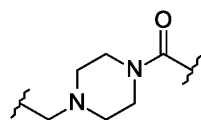


5

or a salt thereof,

wherein R^1 is10 R^2 is selected from the group consisting of PEG_n , a bond, and a peptide, or a combination thereof; n is 1 to 50; and R^3 is a -phenyl-tetrazine group, or a ligation group, wherein the tetrazine is optionally substituted with methyl.

15

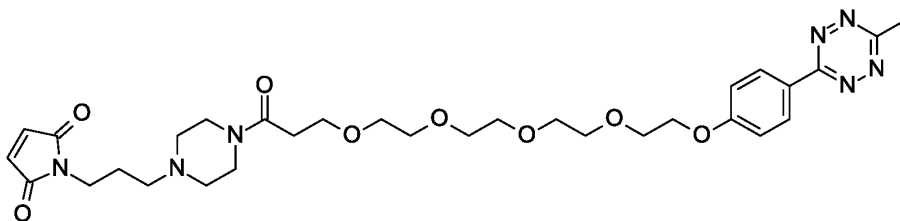
2. The compound according to claim 1 wherein R^1 is

3. The compound according to claims 1 or 2, wherein R^2 is PEG_n , and wherein n is 1 to 10, preferably wherein n is 2 to 6, and more preferably wherein R^3 is a -phenyl-tetrazine group, and wherein the tetrazine is optionally substituted with methyl.

20

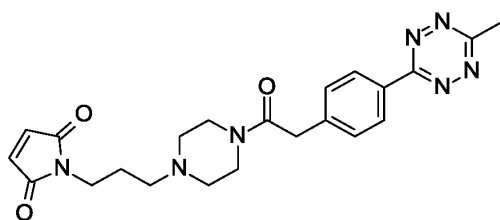
-59-

4. The compound according to any one of claims 1 to 3, wherein the compound is



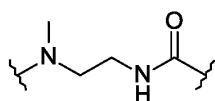
or salt thereof.

5. The compound according to claim 1, wherein R^2 is a bond.
6. The compound according to claim 5, wherein R^3 is a -phenyl-tetrazine group, and wherein the tetrazine is optionally substituted with methyl.
7. The compound according to claims 1, or 5 to 7, wherein the compound is



, or a salt thereof.

8. The compound according to claim 1, wherein R^1 is

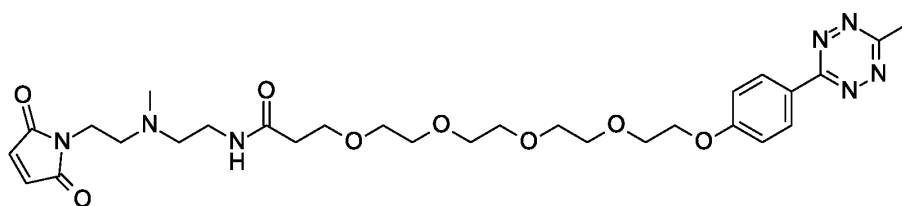


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9. The compound according to claim 8, wherein R^2 is PEG_n , and wherein n is 1 to 10, preferably wherein n is 2 to 6, and more preferably wherein R^3 is a -phenyl-tetrazine group, and wherein the tetrazine is optionally substituted with methyl.
10. The compound according to any one of claims 1, 8, or 9, wherein the compound is

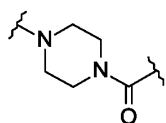
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-60-



or a salt thereof.

11. The compound according to claim 1 wherein R^1 is

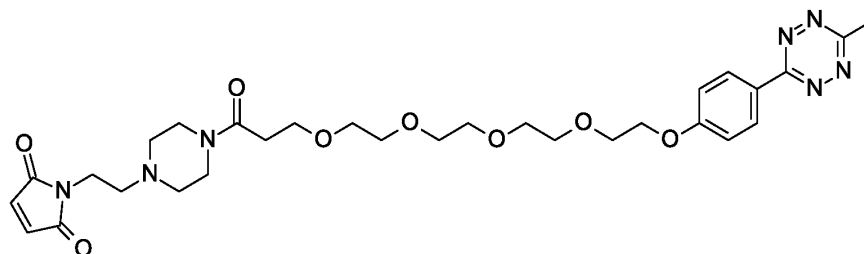


5

12. The compound according to claim 11, wherein R^2 is PEG_n , and wherein n is 1 to 10, preferably wherein n is 2 to 6, and more preferably wherein R^3 is a -phenyl-tetrazine group, and wherein the tetrazine is optionally substituted with methyl.

10

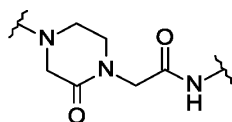
13. The compound according to any one of claims 1, 11, or 12, wherein the compound is



, or a salt thereof.

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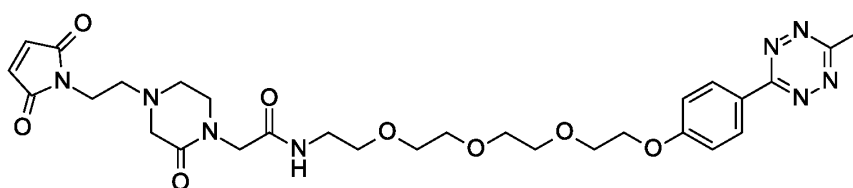
14. The compound according to claim 1, wherein R^1 is



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15. The compound according to claim 14, wherein R^2 is PEG_n , and wherein n is 1 to 10, preferably wherein n is 2 to 6, and more preferably wherein R^3 is a -phenyl-tetrazine group, and wherein the tetrazine is optionally substituted with methyl.

5 16. The compound according to any one of claims 1, 14, or 15, wherein the compound is

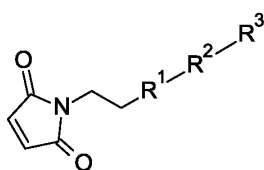


or a salt thereof.

10 17. A method of preparing an antibody-maleimide conjugate comprising: combining a monoclonal antibody (mAb) comprising at least one cysteine and a compound, or a salt thereof, according to any one of claims 1 to 16, to form a reaction mixture.

15 18. The method according to claim 17, wherein the cysteine is an engineered cysteine.

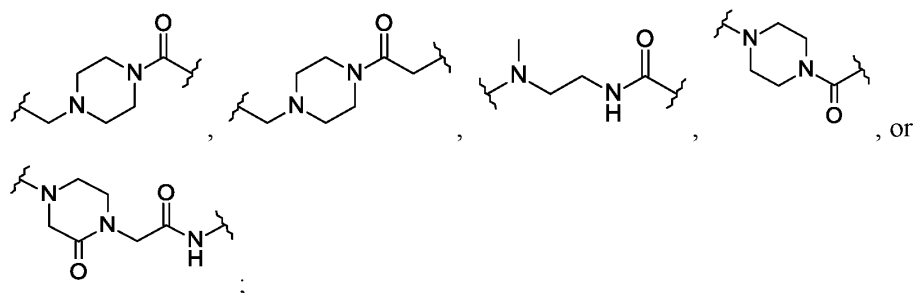
19. Use of a compound of formula:



or a salt thereof, to prepare a pharmaceutical formulation,

20 wherein R^1 is

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R^2 is selected from the group consisting of PEG_n , a bond, and a peptide, or a combination thereof;

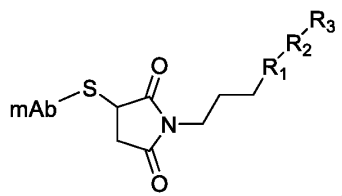
5 n is 1 to 50; and

R^3 is a -phenyl-tetrazine group, or a ligation group, wherein the tetrazine is optionally substituted with methyl, wherein the compound, or a salt thereof, is conjugated to a monoclonal antibody (mAb).

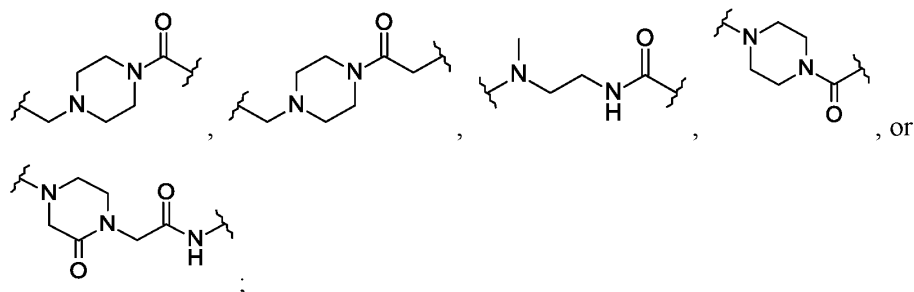
10

20. The use according to claim 19, wherein the cysteine is an engineered cysteine.

21. A conjugate of the formula:



15 or a salt thereof,
wherein R^1 is



R^2 is selected from the group consisting of PEG_n , a bond, and a peptide, or a combination thereof;

20

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n is 1 to 50;

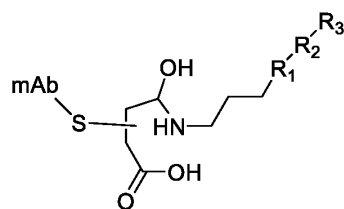
R³ is a -phenyl-tetrazine group, or a ligation group, wherein the tetrazine is optionally substituted with methyl;

mAb is a monoclonal antibody; and

5 S is a sulfur atom from a cysteine residue on the monoclonal antibody.

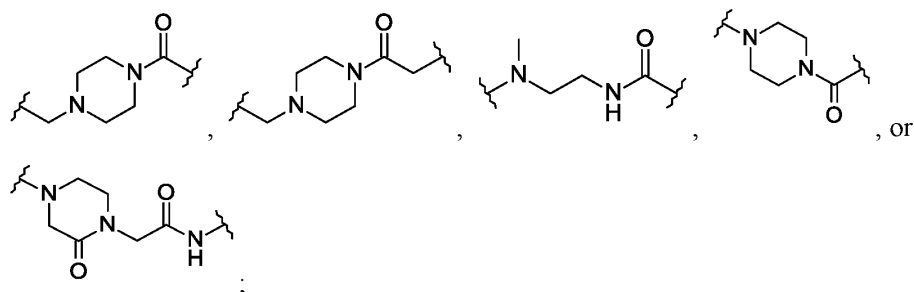
22. The conjugate of claim 21, wherein the cysteine is an engineered cysteine, preferably wherein R³ comprises a drug payload.

10 23. A conjugate of the formula:



or a salt thereof,

wherein R¹ is



15

R² is selected from the group consisting of PEG_n, a bond, and a peptide, or a combination thereof;

n is 1 to 50;

R³ is a -phenyl-tetrazine group, or a ligation group, wherein the tetrazine is optionally substituted with methyl;

20

mAb is a monoclonal antibody; and

S is a sulfur atom from a cysteine residue on the monoclonal antibody.

24. The conjugate of claim 23, wherein the cysteine is an engineered cysteine, preferably wherein R³ comprises a drug payload.